REMARKS

In view of the above amendments and the following remarks, the Examiner is respectfully requested to withdraw the rejections, and allow claims 1, 8, 14-16, 21, 24-26 and 32-54, the currently pending claims. Claims 1 and 35 have been amended. No new matter is added.

The Office Action states that newly submitted Claims 47-54 are directed to an invention that is independent or distinct from the originally claimed invention, and therefore the claims thus presented are withdrawn from consideration. Applicants respectfully traverse.

The Office Action states that Applicants have constructively elected not to prosecute claims directed to a vector requiring two responsive elements controlling the expression of two different transgenes. Applicants respectfully disagree that such a constructive election has been made, but note that if such a restriction is maintained, then "the third sentence of **35 U.S.C. 121** prohibits the use of a patent issuing on an application with respect to which a requirement for restriction has been made, or on an application filed as a result of such a requirement, as a reference against any divisional application, if the divisional application is filed before the issuance of the patent." This nullification of double patenting as a ground of rejection or invalidity in such cases imposes a heavy burden on the Office to guard against erroneous requirements for restrictions.

Applicants respectfully request reconsideration of the Restriction Requirement and rejoinder of Claims 47-52. In the event that such a restriction is made final, applicants reserve the right under 37 C.F.R. 1.144 to petition for reconsideration, which petition may be deferred until after final action on or allowance of claims to the invention elected.

Claims 1, 8, 14-16, 21, 24-26 and 32-46 have been rejected under 35 U.S.C. 112, second paragraph. The Office Action states that "it is unclear how one would know what these particular sequences are given the use of a cell, and such functional language that would have to be tested in a particular cell type".

Without conceding to the correctness of the rejection, Applicants have amended the claims. As described in the specification, an E2F TRE is derived from the E2F1 5' flanking region. In one embodiment, the cell cycle-specific TRE comprises the nucleotide sequence depicted in SEQ ID NO:2. Such sequences, when inserted in an adenoviral vector as set forth in the claims, have certain functional properties. These functional properties include behavior in certain cell types, or in the presence or absence of certain factors. Because the functional properties reside with the sequence of the TRE it is not necessary for the clarity of the claim to include a recitation of the

functional properties. However, in the interest of expediting prosecution of the application, Applicants have amended the claims to include further language related to the function of an E2F TRE.

Similarly, as discussed in previous responses, the sequence of hypoxia responsive elements and binding sites for hypoxia inducible factor-1 are known. Such sequences, when inserted in an adenoviral vector as set forth in the claims, have certain functional properties. These functional properties include behavior in certain cell types, or in the presence or absence of certain factors. Because the functional properties reside with the sequence of the TRE it is not necessary for the clarity of the claim to include a recitation of the functional properties. However, in the interest of expediting prosecution of the application, Applicants have amended the claims to include further language related to the function of HRE and HIF-1.

In view of the above amendments and remarks, withdrawal of the rejection is requested.

Claims 1, 8, 14-16, 21, 25, 26 and 32-46 have been rejected under 35 U.S.C. 103 as unpatentable over either one of Henderson *et al.*, Hallenbeck *et al.*, in view of Walther *et al.*, Dachs *et al.*, Dachs *et al.*, Advani *et al.*, and Parr *et al.*

The Office Action states that Henderson *et al.* disclose conditionally replication competent adenoviruses designed to selectively limit cytolytic replication to specific cell types. It is further stated that "Henderson *et al.* discloses a preferred embodiment comprising replication competent adenovirus comprising a prostate specific antigen (PSA) TRE comprising a cell status specific enhancer (nucleotides from 503 to 2086 of SEQ ID NO:3) and a cell status specific promoter (nucleotides from about 5285 to about 5836 of SEQ ID NO:3) operably linked to the adenovirus E1A promoter (i.e. CN706, p. 33-38; as evidenced by p.49, lines 16-19 of the specification)". Applicants respectfully submit that this characterization of Henderson is not understood. CN706 is an adenoviral vector in which the E1A gene in Ad5 is under transcriptional control of a *PSA*-TRE. CN706 demonstrates selective cytotoxicity toward PSA-expressing cells *in vitro* and *in vivo*. The replication of this virus is specifically sensitive to androgen levels. Therefore, the control of replication of this adenovirus solely depends on the type of cell (i.e. whether a cell is of prostate origin) and not also on its cell-cycle status.

SEQ ID NO:3 in Henderson et al. is the *hKLK2* enhancer. When this sequence is operably linked to an *hKLK2* promoter and a reporter gene, transcription of operably-linked sequences in prostate cells increases in the presence of androgen to levels approximately 30- to approximately 100-fold over the level of transcription in the absence of androgen. This induction is generally

orientation independent and position independent. Again, this TRE is controlled by the presence of androgens, and therefore indicative of the type of cell and not the status of the cell.

In contrast, the present invention relates specifically to adenoviral vectors and methods of use therof, where an adenoviral gene essential for regulation is operably linked to a TRE comprising a hypoxia responsive element; or an E2F-1 TRE. In some embodiments, a composite element containing a cell specific TRE is functionally linked to the hypoxia responsive element or an E2F-1 TRE, the activities of which relate to the status of the cell (hypoxic, cycling), rather than to the type of cell (such as prostate, breast, etc.). One finds that a wide variety of cancers have a high proliferative index (often in cell cycle) or exist under hypoxic conditions, but generally do not synthesize androgens unless derived from an androgen-synthesizing tissue.

The Office Action states that "Hallenbeck *et al.* discloses conditional replication competent adenoviruses to limit cytolytic replication to specific cell types due to operable linking an adenoviral early gene to any one of a number of different tissue or tumor specific promoters". As with Henderson *et al.*, Applicants recognize the contributions to the art made by these earlier filed patents and applications, but respectfully submit that such do not teach the use of specific TREs functionally linked to the hypoxia responsive element, or an E2F-1 TRE, both of which relate to the status of the cell (hypoxic, cycling), rather than the type of cell. It could not have been predicted from the teachings of the prior art that such cell status-specific elements could provide greater specificity of adenoviral replication by using the elements to control expression of an operably linked adenoviral gene.

The advantages provided by the adenoviral vectors of the present invention include enhanced selectivity of in the cytoxicity of the adenovirus, which could not have been predicted from the teachings of the cited art. As evidence of these advantages, Applicants have attached herewith post-filing publications demonstrating the unexpected benefits of the presently claimed methods and compositions.

The first of these publications, (Cuevas et al, Cancer Research 2003 63:6877-84), describes a conditionally replicative adenovirus in which expression of the E1A gene is controlled by a promoter containing multiple hypoxia inducible factor (HIF) recognition elements (HREs). The viral replication and resulting cytolysis was comparable to wild-type in renal cancer cells, but in HIF-defective cells the replication and cytolysis was severely attenuated (from 70% killing by wildtype virus to below 20%). Importantly, this adenovirus displayed tumor-specific cytotoxicity in a

mouse model of tumor cell growth resulting in the complete elimination of 50% of the responding tumors.

Hernandez-Alcoceba *et al.*, Human Gene Therapy 2002 13(14):1737-50 describes oncolytic adenoviruses with linked hypoxia (HRE) and estrogen receptor (ERE) regulated replication; and in some vectors with adenoviral genes also linked to an E2F-responsive promoter. These models may parallel applicants vectors comprising composite HRE and PSA TREs.

One exemplary virus has the E2F-1 promoter incorporated into the E4 region of the human Ad5 genome, together with expression of the adenoviral E1A gene (which is essential for replication) controlled by a minimal HRE/ERE dual-specificity promoter, such that replication occurs in response to estrogens and/or hypoxia. In a xenogenic breast cancer tumor growth assay, this construct when injected reduced tumor size, whereas untreated tumors increased in size by approximately 4-fold. This reference demonstrates that cancer cell specificity and selective cytolysis can be achieved with a replication competent adenovirus comprising an HRE operably linked to an adenovirus gene essential for replication, as set forth in the present claims.

Finally, we include an abstract from the 7th Annual Meeting of ASGT in June 2004. This abstract describes replication-selective oncolytic viruses engineered by replacing the E1A and E1B endogenous promoters with promoters derived from the human E2F-1 and telomerase reverse transcriptase (hTERT) genes, respectively. The results demonstrate that *in vitro*, expression of E1A and E1B genes was highly restricted to Rb-defective and hTERT-positive cancer cells, including Hep3B (hepatocellular carcinoma), LoVo (colorectal carcinoma), A549 (lung cancer), Panc-1 (pancreatic cancer), 253J B-V (bladder cancer), and Hela (cervical cancer). In normal cells, including a lung fibroblast cell line (WI-38) and several other human primary cell lines (HRE, BSMC, PrEC, HMEC and HMVEC-L), no E1A expression could be detected from infection with the E2F-controlled virus. The virus shows activity *in vivo* as well, where strong antitumor activity was seen in NCR nude mice with subcutaneous lung cancer (A549) and bladder transitional cell carcinoma (253J B-V) xenografts. In the latter model, tumors in the treated animals were reduced to 72% of baseline whereas tumors in the control group increased to 944% of baseline. Fully 50% of treated animals had complete regression of the tumor xenograft.

These data demonstrate that state specific transcriptional regulatory elements regulated by hypoxia or by E2F-1 provide for selective replication in tumor cells. The elements can additionally be combined with cell type specific elements. This selectivity could not have been predicted by the teachings of Hallenbeck *et al.*, or Henderson *et al.*, which references each teach the use of cell type

specific TREs, for example, prostate specific regulatory elements. One of skill in the art could not have predicted the success of the presently claimed invention.

Walther *et al.*, Dachs *et al.*, Dachs *et al.*, Advani *et al.* and Parr *et al.* references describe various cell status regulatory elements that are useful in gene delivery vehicles. In other words, the secondary references merely describe particular regulatory elements and this disclosure does not compensate for the lack of teaching in Henderson (WO 97/01358) and Hallenbeck (WO 96/17053) relative to replication-competent adenoviral vectors that exhibits selective cytotoxicity for a cell based on the state of the cell. Thus, one of skill in the art would not arrive at the claimed invention by simply combining the cited references and the combined references do not provide a reasonable expectation of success in practicing the present invention.

In view of the above remarks, withdrawal of the rejection is requested.

CONCLUSION

Applicants submit that all of the claims are now in condition for allowance, which action is requested.

The Commissioner is hereby authorized to charge any other fees under 37 C.F.R. §§ 1.16 and 1.17 which may be required by this paper, or to credit any overpayment, to Deposit Account No. 50-0815, order number CELL-014.

Respectfully submitted,

Date: June 9, 2004

By: Pamela Sherwood

Pamela J. Sherwood, Ph.D.
Registration No. 36,677

BOZICEVIC, FIELD & FRANCIS LLP
200 Middlefield Road, Suite 200
Menlo Park, CA 94025
Telephone: (650) 327-3400
Facsimile: (650) 327-3231

C2L014

ASGT 7th Annual Meeting, Minneapolis, June, 2004

[445] CG5757, an Oncolytic Adenovirus for the Treatment of Retinoblstoma (Rb) Pathway-Defective and Telomerase-Positive Cancers

Yuanhao Li, Natalie Nguyen, Derek Ko, Melinda VanRoey, Gail Colben, Neeraja Idamakanti, Orlena Tam, Trini Arroyo, P.Seshidhar Reddy, Peter Working, De-Chao Yu Cell Genesys, South San Francisco, CA

Replication-selective oncolytic viruses hold promise for the treatment of cancer. Among this novel group of therapeutics are oncolytic adenoviruses engineered with tumor-specific transcriptional response elements (TRE) controlling essential genes. These vectors replicate selectively in cancer cells, leading to expression of toxic viral products and oncolysis mediated by viral replication. We and others have compared different transcriptional control strategies by placing one or more tumor-specific TREs upstream of different viral genes including E1A, E1B and E4. Relocation of the viral packaging signal was also investigated. One such oncolytic virus, CG5757, was generated by replacing the E1A and E1B endogenous promoters with promoters derived from the human E2F-1 and telomerase reverse transcriptase (hTERT) genes, respectively. The E2F-1 promoter is activated in Rb-defective tumor types, a pathway mutated in approximately 85% of all cancers. Likewise, telomerase is aberrantly expressed in over 90% of tumors. CG5757 also has a deletion in the coding region of the E1B 19k gene, a Bcl2-like viral antiapoptotic protein, to increase vector cytotoxicity. CG5757 shows strong tumor selectivity. *In vitro*, expression of E1A and E1B genes was highly restricted to Rb-defective and hTERT-positive cancer cells, including Hep3B (hepatocellular carcinoma), LoVo (colorectal carcinoma), A549 (lung cancer), Panc-1 (pancreatic cancer), 253J B-V (bladder cancer), and Hela (cervical cancer). In normal cells, including a lung fibroblast cell line (WI-38) and several other human primary cell lines (HRE, BSMC, PrEC, HMEC and HMVEC-L), no E1 expression could be detected from infection with CG5757. The transcriptional control of E1 gene expression also correlated with selective viral replication in target cells. CG5757 replicates similarly to wild-type virus in tumor cells, but its replication is, on average, 1,000-times less efficient in normal cells. In a viral cytotoxicity assay, CG5757 destroys tumor cells 100- to 10,000-times more efficiently than normal cells. Comparisons of the cytoxicity of CG5757 in tumor cells versus normal cells (normalized for transduction efficiency with wild-type adenovirus 5) yielded high selectivity indices, some of which were greater than 1000. *In vivo*, strong antitumor activity was seen using CG5757 in *NCR nude* mice with subcutaneous lung cancer (A549) and bladder transitional cell carcinoma (253J B-V) xenografts. With respect to the 253J B-V model, four weeks after treatment the average tumor volume in animals treated with four consecutive daily intratumoral injections of CG5757 ($4x10^8$ particles/mm^3 of tumor) decreased to 72% of baseline while the control group had an increase to 944% of baseline. Furthermore, 50% of treated animals had complete regression of the 253J B-V tumor xenografts. The potential therapeutic efficacy of such dual promoter controlled oncolytic adenoviruses in cancers that are Rb-defective and hTERT-positive has been demonstrated.

CGR 014

[CANCER RESEARCH 63, 6877–6884, October 15, 2003]

Specific Oncolytic Effect of a New Hypoxia-Inducible Factor-Dependent Replicative Adenovirus on von Hippel-Lindau-Defective Renal Cell Carcinomas[1]

Yolanda Cuevas, Rubén Hernández-Alcoceba, Julian Aragones,[2] Salvador Naranjo-Suárez, María C. Castellanos, Miguel A. Esteban, Silvia Martín-Puig, Manuel O. Landazuri, and Luis del Peso[3]

Servicio de Inmunología, Hospital de la Princesa, Universidad Autónoma de Madrid, 28006 Madrid [Y. C., J. A., S. N-S., M. C. C., M. A. E., S. M-P., M. O. L., L. d. P.], and Departamento de Medicina Interna, Universidad de Navarra/Fundación de Investigaciones Medico-Aplicadas, Edificio los Castaños, 31008 Navarra [R. H-A.], Spain

ABSTRACT

Mutations in the von Hippel-Lindau (*VHL*) tumor suppressor gene are responsible for a hereditary cancer syndrome characterized by high susceptibility to hemangioblastomas of the retina and central nervous system, pheochromocytomas, and renal cell carcinomas. In agreement with its role as a tumor suppressor, the vast majority of spontaneous clear cell carcinomas of the kidney present loss of heterozygosity at the *VHL* locus. Recently, it has been shown that VHL works as the substrate recognition component of an E3 ubiquitination complex that targets the hypoxia-inducible factor (HIF) for proteosomal degradation. Under normal oxygen tension, the half-life of HIF transcription factors is extremely short because of its high degradation rate by the proteasome, resulting in undetectable HIF activity in normal cells. However, in VHL-deficient tumor cells, the HIF transcriptional pathway is constitutively activated because of impaired ubiquitination of this transcription factor. To target VHL-deficient tumors, we have exploited this feature to develop a conditionally replicative adenovirus (Ad9xHRE1A), the replication of which is HIF dependent. In this new oncolytic adenovirus, the expression of the E1A gene is controlled by an optimized minimal promoter containing HIF recognition elements. Here, we show that the induction of the E1A gene, as well as the viral replication and cytolytic effect of Ad9xHRE1A, are dependent on HIF activity. As a consequence, this virus efficiently kills VHL-deficient cells both *in vitro* and *in vivo*, as well as cells growing under hypoxic conditions. These data suggest that Ad9xHRE1A could be used as a highly specific therapy for VHL-deficient cancers and probably many other tumors that show extensive hypoxic areas or increased HIF activity by genetic alterations other than *VHL* loss.

INTRODUCTION

Because oxygen is essential for the survival of most cell types, they respond to changes in oxygen tension by the induction of adaptive responses aimed to restore oxygen supply and maintain energy balance. The response to decreased oxygen is mediated by the activation of a specific set of genes, most of them under the control of the HIFs.[4] HIF transcription factors are heterodimers of a constitutively expressed β subunit (also known as aryl receptor nuclear translocator) and an oxygen-regulated α subunit (1). The HIF-β subunit, as well as the three different HIF-α subunits identified to date, belong to the basic helix-loop-helix-Per/aryl receptor nuclear translocator/Sim transcription factors. Under normal oxygen tension, HIF-α is undetectable because of a high degradation rate by the proteasome (1). When oxygen is limiting, its half-life increases and HIF-α protein accumulates, allowing its interaction with HIF-β and the transcriptional activation of target genes needed for the cellular adaptation to hypoxia (1). The observation that cells deficient for the tumor suppressor gene *VHL* overexpressed HIF target genes such as VEGF led to the identification of VHL protein as a critical element of the oxygen sensing pathway. VHL protein is the substrate recognition component of an E3 ubiquitin ligase complex that targets HIF for proteosomal degradation. However, VHL/HIF-α interaction depends on the hydroxylation of two specific proline residues on HIF-α, a reaction catalyzed by a novel family of 2-oxoglutarate-dependent dioxygenases (1). Because these enzymes require molecular oxygen for their catalytic reaction, it is accepted that they act as the oxygen sensors that directly control HIF stability in response to oxygen variations. As predicted by this model, loss of VHL results in HIF-α accumulation and activation of target genes, regardless of the presence or absence of oxygen.

Long before the role of pVHL on HIF regulation was known, the gene encoding for pVHL was identified as a tumor suppressor involved in a rare hereditary cancer syndrome (VHL syndrome) characterized by high frequency of hemangioblastomas, pheochromocytomas, and clear cell renal carcinomas. In agreement with its role as a tumor suppressor, *VHL* is lost in the vast majority of sporadic clear cell carcinomas of the kidney. It is currently unclear whether HIF-α up-regulation is the major factor responsible for the generation of a full transformed phenotype after *VHL* loss (2, 3), but it is generally accepted that HIF target genes contribute to tumor progression in VHL-negative cells. For example, the induction of VEGF expression by HIF plays a critical role in promoting tumor-mediated angiogenesis, and accordingly, clear cell carcinomas are highly vascularized and metastatic. In addition, it has been recently described that the HIF activation that occurs in VHL-deficient cells results in overexpression of metalloproteinases that promote enhanced migration (4).

Current cancer treatments depend on the use of drugs that have serious side effects and are often of limited value for the control of the disease. This is particularly true for clear cell carcinomas of the kidney (5). Thus, the development of new strategies for the treatment of these and other cancers is needed. One of such strategies that is particularly promising is the use of modified adenoviruses that replicate and complete their lytic cycle preferentially in cancer cells (CRADs; Refs. 5, 6). In this approach, the initial administration of the virus kills the infected cells, and a new viral progeny is released that can repeat the process and amplify the oncolytic effect until the tumor is eliminated. One method to achieve tumor specificity of viral replication is based on the transcriptional control of genes that are critical for virus replication such as E1A or E4. The promoters of these genes are substituted by regulatory sequences that are preferentially activated in cancer cells. We and others (6, 7) have successfully devel-

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[2] Present address: Center of Transgene Technology and Gene Therapy, Vlaams Interuniversitair Instituut voor Biotechnologie, Kuleuven Campus Gasthuisberg O&N, Herestraat 49, B-3000 Leuven, Belgium.

[3] To whom requests for reprints should be addressed, at Phone: 34-91-5202371; Fax: 34-91-5202374; E-mail: lpeso.hlpr@salud.madrid.org.

[4] The abbreviations used are: HIF, hypoxia-inducible factor; MOI, multiplicity of infection; HRE, hypoxia response element; Q-RT-PCR, quantitative real-time PCR; VEGF, vascular endothelial growth factor; CRAD, conditionally replicative adenovirus; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; pfu, plaque-forming unit(s); VHL, von Hippel-Lindau; RCC, renal cell carcinoma; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; pVHL, VHL protein; pRb, retinoblastoma protein (product of the RB tumor suppressor gene); HUVEC, human umbilical vascular endothelial cells.

oped CRADs for the targeting of tumors with specific genetic alterations. The constitutive activation of HIF transcription factors that is intrinsically associated to *VHL* loss makes tumors harboring this alteration suitable targets for a CRAD in which viral replication is under the control of HIF. In fact, we have previously developed a series of CRADs in which replication is controlled by hybrid promoters containing estrogen response elements and HREs to target the hypoxic areas of breast tumors (7–9). In one of these viruses (AdEHE2F), the E4 coding region is under the control of an E2F-1-responsive promoter (7). The activity of E2F transcription factors is required for cell cycle progression, and in most tumor cells, the E2F activity is up-regulated because of diverse alterations in the pRb tumor suppressor pathways, which normally have an inhibitory effect on E2F activity. In addition, expression of the adenovirus E1A protein induces E2F activity by sequestering pRb. In this type of CRAD, the regulation of E4 by E2F activity restricts its expression to proliferating cells, including tumors. However, because E1A protein interferes with pRb, E1A induction ensures E2F activation and E4 expression (7, 10). Thus, regulation of E4 region by E2F is not intended as an independent level of regulation but rather as an amplification loop upon E1A activation in tumor cells.

Here, we describe the generation of a new version of HIF-dependent CRAD (Ad9xHRE1A) to target tumor cells with constitutive activation of the HIF pathway such as VHL-deficient renal carcinomas. We have maintained the E2F-1 promoter in the E4 region of this virus for the reasons discussed above. On the other hand, for the control of the E1A region, we have used an optimized minimal promoter because this is the single most important regulatory element for the replication of adenovirus. This is an artificial promoter that contains nine tandem copies of the HRE for binding of HIF. The simplicity of its sequence reduces the chances that other transcription factors will bind to the promoter and abolish its specificity, especially in the context of an adenovirus genome. We have characterized in detail this new CRAD and have found a tight regulation of the E1A expression that closely correlates with its ability to replicate and kill VHL-deficient cancer cells. Finally, we provide data showing antitumor effect of this virus *in vivo*.

MATERIALS AND METHODS

Cell Culture and Reagents. pVHL-deficient 786-O cell clones stably expressing wild-type *VHL* (WT-10), truncated *VHL* (1-115), or empty plasmid (PCR3) were kindly provided by William Kaelin (Dana-Farber Institute, Boston, MA). Parental *VHL*-negative RCC4 cells (herein described as RCC4−) and the corresponding *VHL* stable transfectants (RCC4+) were provided by Dr. Patrick H. Maxwell (Imperial College, London, United Kingdom). Parental VHL-negative RCC10 cells and a clone derived by stable transfection of *VHL* (VHL53) were provided by Dr. Karl H. Plate (Johann Wolfgang Goethe University, Frankfurt, Germany). Parental VHL-defective UM-RC6 cells and the stable *VHL* transfectant, UM-RC6 3-4 (herein referred as 3-4), were provided by Dr. Michael I. Lerman and Dr. Sergey V. Ivanov (Laboratory of Immunobiology, National Cancer Institute, Frederick, MD). WT-10-PP13 clone derives from WT-10 cells upon stable expression of P402A, P564G mutant HIF-1α; it was generated by Silvia Martín *et al.* and will be described in more detail in a manuscript in preparation.[5] All cells were maintained in RPMI 1640 with Glutamax-I (Life Technologies, Inc.). For WT-10, 1-115, PRC3, WT-10-PP13, VHL +53, and RCC4+, G418 sulfate (100 mg/ml; Promega) was added to culture media. HeLa and IMR-90 human fibroblast were maintained in DMEM (Life Technologies, Inc.). In all cases, culture media were supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin and 10% fetal bovine serum. Human umbilical vascular endothelial primary cells, were isolated and grown as previously described (11). Hypoxia (1%) was induced by culture of cells in an *in vivo* 400 hypoxia workstation (Ruskinn Technology). Deferoxamine was purchased from Sigma (St. Louis, MO), anti-HIF-1α from Tranduction Laboratories, anti-HIF-2α/EPAS from Novus Biologicals (Littleton, CO), and anti-α-Tubulina from Sigma.

Construction of the Ad9xHRE1A Virus. The virus was constructed by promoter replacement of the E1A and E4 regions after a previously described method (7). Briefly, we amplified the 9XHIF promoter by PCR from the plasmid p9xHIF-Luc (12), using the primers 5′-TCAGTGCTAGCTTCGAAGCCATATCACATTTGTAGAGG-3′ and 5′-CCTAGAGATCTTTCGAACAAGCTTGACCACACTTCC-3′. These primers introduce *Bst*BI restriction sites flanking the 9XHIF construct for its introduction into the E1A promoter region of the plasmid pSEHE2F. This plasmid contains unique restriction sites flanking the promoter regions of E1A (*Bst*BI sites) and E4 (*I-Ceu*I and *Swa*I sites). In the latter region, we maintained the E2F-1 promoter (bp, −218 to +51; Ref. 7) that was obtained by PCR from human genomic DNA using the primers 5′-TACTGTAACTATAACGGTCCTAAGGTAGCGTGGTACCATCCGGACAAAGCC-3′ and 5′-TAAGTATTTAAATGGCGAGGGCTCGATCCCGC-3′. The new plasmid (named pSHIFE2F) was digested with *Pac*I to liberate the modified viral genome. After ethanol precipitation, 10 μg were used to transfect 293E4pIX cells growing in 10-cm dishes by the calcium phosphate method. The cells were treated with 1 μM dexamethasone until the cytopathic effect was observed. Individual viral plaques were isolated from infected monolayers growing under semisolid medium. The modification in the E1A and E4 promoter regions were verified by PCR using specific primers. The viruses were amplified in A549 cells growing in the presence of 100 μM $CoCl_2$ to mimic hypoxic conditions, purified using CsCl gradients, and desalted in G-50 Sephadex columns. Titration was done after the plaque-forming assay method.

Western Blot. The level of HIF-1α, HIF-2α/EPAS and α-tubulin protein expression was determined by immunoblotting. Immunolabeling was detected by enhanced chemiluminescence (Amersham Pharmacia Biotechnology, Piscataway, NJ) and visualized with a digital luminescent image analyzer (Fujifilm LAS-1000 CH).

Measure of HIF-Dependent Transcriptional Activity. Cells were cotransfected by lipofection with the HIF-responsive firefly luciferase reporter (12) and a Renilla luciferase expression plasmid at a 40:1 ratio. We used Lipofectamin (Roche Applied Science) for 786-O and UM-RC6 lines and Superfect (Qiagen) for RCC4 and RCC10 lines. Cell lines were transfected as a pool for either 24 h (Lipofectamin) or 10 h (Superfect) in 100-mm culture dishes, after transfection cells were split into 24-well plates and, 20–24 h after plating, submitted to hypoxia (1%) or left at normoxia for 10 h. Finally, cells were harvested and firefly and Renilla luciferase activities were determined using a dual luciferase system (Promega). Firefly luciferase activity was normalized based on the Renilla luciferase activity. The average and SD of triplicate samples for each condition is represented. HeLa cells were always included as a control for reporter inducibility (data not shown).

Q-RT-PCR. The levels of VEGF mRNA and E1-A mRNA were determined by Q-RT-PCR. Immediately after treatment, cells were harvested into 1 ml of Ultraspec reagent (Biotecs, Houston, TX). Total RNA was extracted, quantified, and integrity tested by gel electrophoresis. One μg of total RNA from each sample was retrotranscripted to cDNA (Improm-II reverse transcriptase; Promega). One to 3 μl of cDNA samples were used as template for amplification reactions carried out with the LC Fast Start DNA master Sybr Green I kit (Roche Applied Science, Mannheim, Germany) following manufacturer instructions. PCR amplifications were carried out in Light Cycler System (Roche Applied Science) and data analyzed with LightCycler software 3 version 3.5.28 (Idaho Technology, Inc.). For analysis purposes, the amplicon for each of the analyzed genes was cloned, and known amounts of the cloned product were used to generate a standard curve. The number of copies of the interest gene in each sample was extrapolated from the corresponding standard curve by the indicated software. For each sample, duplicate determinations were made, and the gene copy number was normalized by the amount of β-actin on the same samples. The primers used in this study are (5′-3′): VEGF-A, forward (TGCCAAGTGGTCCCAG) and reverse (GTGAGGTTTGATCCGC); β-actin, forward (CCCAGAGCAAGAGAGG) and reverse (gTCCAGACGCAGGATG); and E1A, forward (CTTGTCATTATCACCGGAG) and reverse (TCCGTACTACTATTGCATTCT).

[5] S. Martín-Puig, E. Temes, R. Martín, J. Aragones and M. O. Landázuri. Pro564 and Pro402 are essential for the full induction of HIF1a by hypoxia, manuscript in preparation.

Adenoviral Infection and Cell Viability Assays. Cells were plated at 10^5 cells/well in 24-well plates before infections. Twenty-four h later, the media were removed and replaced with 2% media. Cells were infected by exposure to adenovirus preparations diluted to the indicated MOIs in 200 μl of RPMI supplemented with 2% FCS. After 1 h incubation with frequent gentle shaking, 300 μl of fresh media were added, and 48 h after infection, an additional 0.5 ml of fresh media were added to each well. During the course of the experiment, 0.5 ml of media were removed from cultures and replaced with 0.5 ml of fresh media every 48 h.

Cell viability was determined 7–10 days after infections by the MTT assay (Sigma). At the time of analysis, 25 μl of Thiazolyl blue (MTT, 5 mg/ml) solution were added to the cell cultures in 0.5 ml of medium. Four h later, media were removed and precipitated MTT salts solubilized into 150 μl of 0.04 N HCl diluted in isopropanol. Product formation was quantified by reading solution absorbance at 550 nm.

Adenovirus Replication Assay. Cells were plated in 24-well plates, and 24 h later, cells were infected with Ad-5WT or Ad-9xHRE1A at a MOI of 10 pfu/cell. Twenty-four h after infections, cells were washed four times with fresh medium to removed virus excess. Eight days later, the plates were centrifuged at 4°C for 10 min at 4000 × *g* to remove floating cells, and cell culture supernatants were collected. Viral titer was calculated by infection of HEK293 cells with 200 μl of the supernatants and 300 μl of fresh medium supplemented with 2% serum. Infected HEK293 cells were identified 48 after infection by immunostaining against viral protein (hexon) with the Adeno-X Rapid Titer Kit (BD Biosciences Clontech, Palo Alto, CA) following manufacturer instructions.

Adenoviral Infectivity Assays, X-Gal Staining. Cells were seeded on 24-well plates at a density of 2×10^5 cells/well. Twenty-four h after plating, cells were infected with Adeno-X-LacZ (BD Biosciences Clontech), an adenovirus that expressed the β-galactosidase gene at a MOI of 50 pfu/cell. Forty-eight h after exposure to Adeno-X-LacZ, infected cells were identified on the cell monolayer by fixing for 20 min in a solution containing 50% (w/w) glutaraldehyde in PBS and staining with the β-galactosidase substrate X-Gal. The staining solution contained X-Gal (1 mg/ml), 2 mM $MgCl_2$, 4 mM $K_3Fe(CN)_6$, and 4 mM $K_4Fe(CN)_6$ (Sigma) in PBS. The number of blue cells and unstained cells/field was counted, and percentage of infectivity was determined.

***In Vivo* Assays in Nude Mice.** The antitumor effect of Ad-9xHRE1A was tested in human tumor xenografts implanted in 6–8-week-old Swiss-nu/nu mice (Charles River, ICO:Swiss-FoxN1nu). A total of 1.3×10^7 1-115 (786-O) cells was resuspended in PBS and injected s.c. in the back flanks. When tumors reached a volume of ~200 mm^3, mice were treated by intratumoral injection of Ad-9xHRE1A (1.6×10^8 pfu in 50 μl of glycerol/BSA buffer) or glycerol/BSA buffer (100 mM Tris (pH 8.1), 100 mM NaCl, 1% BSA, and 50% glycerol) alone (control group). One day later, treatments were repeated. Tumor volume was measured weekly and calculated using the equation: $D \times (d)^2/2$, where D and d are the major and minor diameters, respectively. Sixty days after treatments, mice were sacrificed and tumors excised and weighted.

Animals were maintained under specific pathogen-free conditions and treated according to a protocol approved by the Universidad Autonoma de Madrid Animal Care Committee.

RESULTS

Characterization of Clear Cell Renal Carcinoma Cell Lines. We chose several different cell lines derived from four independent human clear cell carcinomas of the kidney: 786-O (13, 14); RCC10 (15); UM-RC6 (16, 17); and RCC4 (18) cells. 786-O renal carcinoma cells lack functional pVHL and, as consequence, overproduce HIF-2α (18) and HIF target genes such as VEGF (19). Several cell lines were obtained by stable transfection of 786-O cells with plasmids encoding wild-type VHL protein (WT-10), truncated inactive VHL protein (1-115), or empty plasmid (PRC3). These cell lines have been widely used for the study of pVHL and HIF-α function (18). Similarly, RCC10, UM-RC6, and RCC4 cells derive from human renal carcinomas lacking functional pVHL and present constitutively elevated HIF-1α protein under normoxia. Finally, the VHL53, 3-4, and RCC4+ cell lines derive from RCC10, UM-RC6, and RCC4−, respectively, after stable transfection with wild-type *VHL* gene. As shown in Fig. 1*A*, all pVHL-defective cell lines (PRC3, RCC10, UM-RC6, and RCC4−) express significant amounts of HIF-α in the presence of oxygen (normoxia) that are not further increased after exposure to hypoxia. In agreement with previous studies (13–18), after restoration of normal VHL function, HIF-α protein was only detected under low oxygen tension (hypoxia) or in the presence of hypoxia mimetic chemical agents such as deferoxamine (Fig. 1*A*).

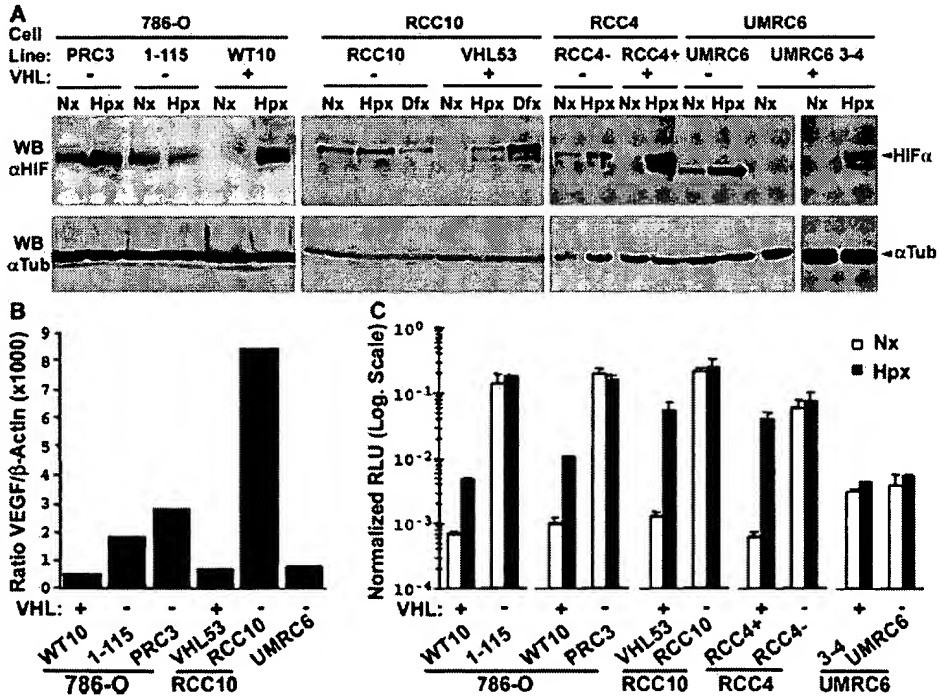

Fig. 1. Characterization of the renal cancer cell lines used in this work. *A*, cells were grown on 6-well plates and transferred to 1% oxygen atmosphere (Hpx, hypoxia) or left at normoxic conditions (Nx) for 12 h. Where indicated, cells were exposed to the hypoxia-mimetic compound deferoxamine (Dfx, 380μM) for the same period of time. After treatments, cellular protein extracts were fractionated by SDS-PAGE, and the level of HIF-α protein was determined by immunoblot. As a control, the level of α-tubulin on each sample is also shown. In all of the cases, except for 786-O cells, HIF-1α expression is shown. 786-O cells do not express detectable amounts of HIF-1α. Therefore, in this case, HIF-2α was determined instead of HIF-1α. *B*, the abundance of VEGF-A mRNA was determined by Q-RT-PCR and represented after normalization by the amount of β-actin. *C*, cells were transiently transfected with and HRE-driven firefly luciferase reporter plasmid together with a Renilla luciferase expression plasmid and then transferred to Hx (1% O_2) or left at Nx for 8–10 h. The average firefly luciferase activity, normalized to the Renilla luciferase activity, in duplicate samples is represented. The results shown in *A–C* are representative of at least two independent experiments.

Importantly, the overexpression of HIF-α under normoxic conditions found in VHL-defective cell lines correlates with the induction of HIF target genes such as VEGF in all cases, except UM-RC6 cell line (Fig. 1*B*). The unexpected behavior of UM-RC6 cell line regarding VEGF production has been previously noticed (17) and suggests that although HIF-α is overexpressed under normoxia in this cell line (Fig. 1*A*), it may be nonfunctional for the induction of some target genes such as VEGF.

Next, we investigated the activity of HIF on different cell lines. To this end, we transfected them with a reporter construct that express the firefly luciferase gene under the control of a rat prolactime minimal promoter downstream of nine tandem copies of the HRE found in the VEGF promoter (12, 20). To prevent unspecific expression of the reporter gene, two tandem SV40 polyadenilation signals were included upstream of the promoter control elements (12). Previous experiments with this construct indicate a tight hypoxia-dependent regulation of the reporter gene (12, 20). Fig. 1*C* shows that the reporter gene expression is elevated under normoxic conditions in all VHL-deficient cells, except UM-RC6, compared with the VHL-competent cells. Moreover, HIF-regulated luciferase expression was induced by hypoxia only in those cell lines expressing functional pVHL. Again the 3-4 cell line, which was generated by stable transfection of *VHL* into UM-RC6 cells, was exceptional in that hypoxia did not up-regulate HIF activity (Fig. 1*C*), although expression of pVHL in 3-4 cells restores HIF-α protein regulation by oxygen (Fig. 1*A*).

Thus, all VHL-deficient cells present deregulated HIF protein expression that results in increased HIF activity, with the exception of the UM-RC6 cell line. Forced expression of pVHL in these renal carcinoma cells restores normal HIF regulation. It is unclear why UM-RC6 cells, despite presenting high basal levels of HIF protein, are unable to induce HRE-dependent reporters or up-regulate VEGF mRNA. Future work will address this issue. Regardless of the mechanism, the deficient induction of HRE-driven genes in this VHL-deficient cell line provides a unique opportunity to test the specificity of HIF-dependent therapeutic strategies.

Sensitivity of Clear Cell Renal Carcinoma Cells to Adenovirus Infection. Adenoviruses bind to the surface of cells mainly through the coxsackie adenovirus receptor, and the internalization requires the participation of integrins $\alpha v\beta 3$ and $\alpha v\beta 5$. Because loss of pVHL has been related to alterations on integrin function (21), we decided to study whether VHL deficiency affected adenovirus infection. For this purpose, cells were infected with an E1-defective adenovirus expressing the *LacZ* gene under the control of a cytomegalovirus promoter. After infections, cells were fixed and processed to determine β-galactosidase-expressing cells. As shown in Fig. 2, there is a wide range of sensitivity to adenovirus infection among the different cell lines, WT-10, RCC10, UM-RC6, and HeLa (data not shown) being the most sensitive ones. However, we found no correlation between VHL status and sensitivity to adenovirus infection (Fig. 2*B*). It is of note that there is a large difference in the sensitivity to adenovirus infection between the cell lines derived from the same renal cancer cells such as 1-115 and WT-10 (Fig. 2*B*).

Generation of Ad9xHRE1A. For the construction of Ad9xHRE1A, we used an adenoviral backbone designed to facilitate the substitution of E1A and E4 promoters by tumor-specific promoters (7). In the Ad9xHRE1A virus, the E1A coding region is under the control of a minimal promoter containing nine tandem copies of a HRE derived from the VEGF promoter. This artificial sequence was placed upstream of a TATA sequence from the rat prolactine promoter. In addition to the sequence already contained in the adenoviral backbone that insulates the modified E1A promoter (7), we included the SV40 polyadenylation signal to further decrease the possibility of E1A expression from transcripts initiated upstream de HRE- controlled promoter (Fig. 3*A*). The complete sequence of this region is shown in Fig. 3*B*. Note that E1A is under the control of a promoter identical to the one used for the reporter assays in Fig. 1*C*. As in previous CRADs generated by our group (7), the E4 coding region was placed under the control of the E2F-1 promoter (Fig. 3*A*). In this case, the promoter does not provide a genuine second level of control on viral replication, but it contributes to the attenuation of the virus in normal cells and amplification of viral replication upon E1A induction (7).

To test the efficiency and specificity of the 9xHRE promoter in the control of E1A expression, we infected different cell lines with wild-type or Ad9xHRE1A and determined the level of E1A mRNA by Q-RT-PCR after infection. As shown in Fig. 3*C*, there are large differences in E1A mRNA expression among the different cell lines infected with wild-type adenovirus. This correlates with the different sensitivity of each cell line to infection (Fig. 2). Importantly, the expression of E1A mRNA upon infection with Ad9xHRE1A in cells with deregulated HIF activity such as 1-115, PCR-3, and RCC10 is similar or even higher than the expression of E1A gene from wild-type adenovirus (Fig. 3*C*). In contrast, in cells were VHL activity was restored (WT-10) in normal cells (IMR90) or cells with impaired HIF activity (UM-RC6), the expression of E1A upon infection with Ad9xHRE1A is dramatically reduced as compared with infection with the wild-type adenovirus (Fig. 3*C*). The doses of virus used in this experiment were deliberately high (MOI of 100 pfu/cell). Even at these high viral doses, E1A mRNA levels in VHL-positive cells infected with Ad9xHRE1A were reduced to 10% of the values obtained after infection with wild-type virus (Fig. 3*C*). Thus, basal E1A expression from Ad9xHRE1A upon infection of nontarget cells is very low. The low expression of E1A in UM-RC6 cells is of particular interest because it suggests that the induction of E1A observed on the other pVHL-deficient cells (1-115, PCR3, and RCC10) is mediated by

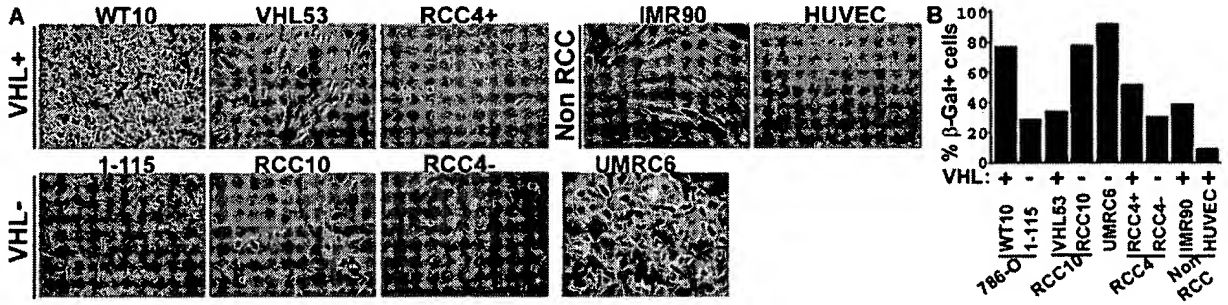

Fig. 2. Susceptibility to adenovirus infection of the different cell lines used in this study. Cells were grown in 24-well plates until confluent and then infected with Ad-LacZ at a MOI of 50 pfu/cell. Forty-eight h after infection, cells were fixed and processed to determine β-galactosidase activity. A representative microphotograph of each cell line is shown. The number of β-galactosidase positive cells in several independent fields was determined, and it is represented as percentage of total number of cells.

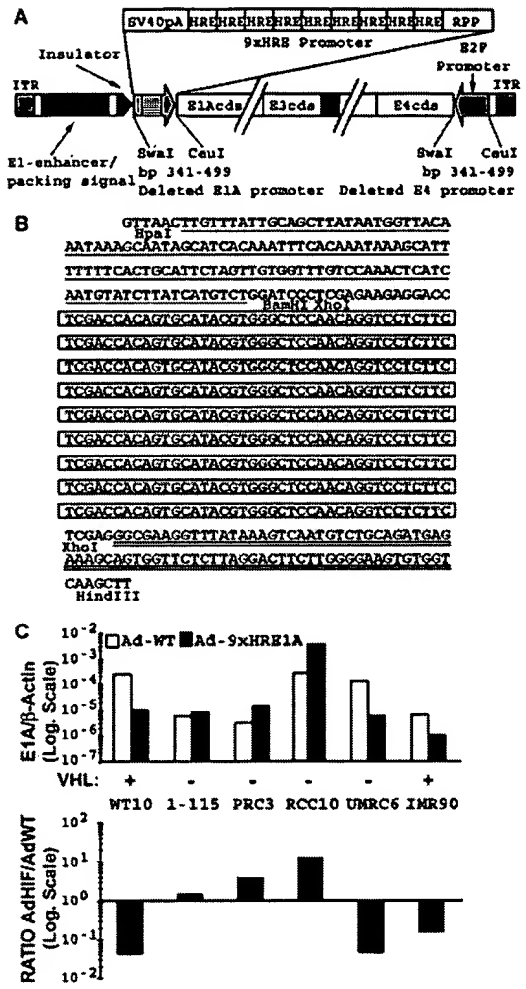

Fig. 3. Characterization of Ad9xHRE1A. *A*, schematic representation of the modifications introduced in the viral genome. An insulator sequence (Insulator) and a polyadenilation signal from SV40 (SV40pA) were placed between the overlapping E1A enhancer/ packaging signals and the E1A promoter. The wild-type E1A promoter was substituted by an artificial minimal promoter containing nine HREs upstream of a TATA sequence from the rat prolactine promoter (RPP). The E4 promoter was substituted by the E2F-1 promoter. ITR, inverted terminal repeats; cds, coding sequence. *B*, the complete sequence of the artificial promoter is shown. Underlined sequence corresponds to the polyadenilation signal; *double underlined sequence* derives from the rat prolactine promoter; the nine HREs from VEGF-A promoter are *boxed*. The restriction sites are indicated. *C*, cells were infected with Ad9xHRE1A or wild-type adenovirus at a MOI of 100 pfu/cell. Twelve h after infection, RNA was obtained from cells, and the level of E1A mRNA in samples was determined by Q-RT-PCR. The amount of E1A mRNA, normalized by β-actin content, is represented in the *top graph*. The *bottom graph* shows the ratio between the normalized E1A expression levels in cells infected with Ad9xHRE1A (AdHIF) and wild-type virus (AdWT). Similar results were obtained in three independent experiments.

the elevated HIF activity and not because of other alterations associated to *VHL* loss.

Oncolytic Effect of Ad9xHRE1A on RCCs. Because the expression of E1A from Ad9xHRE1A is tightly regulated and restricted to cells with increased HIF activity, it is expected that its cytolytic effect would be restricted to this type of cells. To verify this hypothesis, we infected a panel of cells with wild-type adenovirus or Ad9xHRE1A at different MOIs and determined the number of viable cells remaining 8–10 days after infection (see "Materials and Methods"). As shown in Fig. 4*A*, Ad9xHRE1A efficiently eliminates VHL-deficient renal cancer cells with increased HIF activity (PRC3, 1-115, RCC10, and RCC4−) as efficiently as wild-type virus. Importantly, Ad9xHRE1A is attenuated in the VHL/HIF-defective UM-RC6 cells, although they are highly susceptible to adenovirus infection as demonstrated by the effect of the wild-type virus (Fig. 4*A*). In support of the specificity of Ad9xHRE1A, the oncolytic effect of this virus was dramatically reduced as compared with wild-type virus in renal cancer cells where pVHL function had been restored (WT-10, VHL53+, and RCC4+) and tumor cells where the HIF regulation is intact (HeLa cells) or normal cells such as IMR-90 fibroblast and primary HUVEC (Fig. 4*A*).

In Fig. 4*B*, we represent the survival of cells infected with the Ad9xHRE1A virus. Each cell type was infected using the MOI that causes >70% mortality when the wild-type adenovirus was used. The results of several independent experiments are represented, demonstrating that the effect of the Ad9xHRE1A virus was highly reproducible. These experiments demonstrate that Ad9xHRE1A is very efficient and specific in the killing of cells with elevated HIF activity. To verify that this selective killing was attributable to the replication and production of competent viral particles, we tested the supernatant

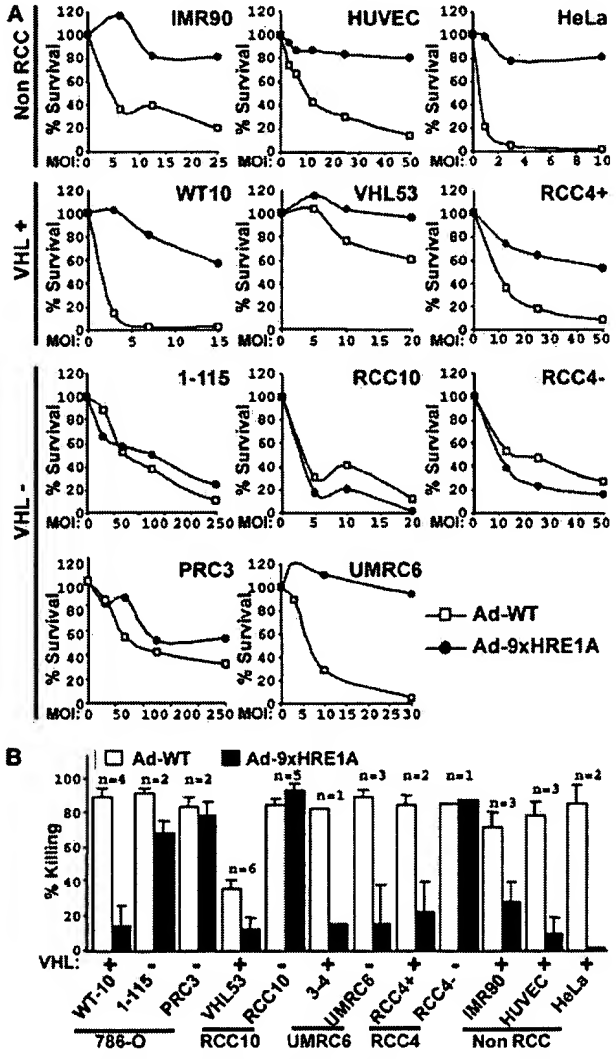

Fig. 4. Oncolytic effect of Ad9xHRE1A1A on different cell lines. *A*, cells were plated on 24-well plates, and 24 h later, they were infected with Ad9xHRE1A or wild-type adenovirus at the indicated MOIs. The range of MOIs used for each cell line was adjusted according to its susceptibility to adenovirus infection (Fig. 2). Seven to 10 days after infection, the number of surviving cells was assessed by the ability to reduce MTT. The average values of duplicate determinations are represented as percentage of the value obtained for uninfected cultures. The results shown are representative of two to six independent experiments. *B*, the results from *n* independent experiments as the one shown in *A* are represented as the mean ± SE. To compare different cell lines, we chose for each case the smallest MOI required to kill >70% of the cells with the wild-type adenovirus.

of infected cultures for the presence of viable viral particles. VHL-deficient cells (RCC10 and UM-RC6) as well as VHL-competent cells (VHL53+) were infected with Ad9xHRE1A or wild-type adenovirus, and 10 days later, culture supernatants were collected and used to infect HEK293 cells. Forty-eight h after exposure to culture supernatants, infected HEK293 cells were identified by immunostaining using antiadenovirus antibodies (hexon protein). As shown in Fig. 5, supernatants of cells infected with wild-type adenovirus contain a large number of infective particles regardless of the cellular source of the supernatant. In contrast, infective viral particles were rescued from RCC10 supernatants infected with Ad9xHRE1A but not from VHL53+ or UM-RC6 cell lines (Fig. 5). Thus, the specific oncolytic effect of Ad9xHRE1A in VHL-deficient cells is because of its restricted replication and amplification in target cells.

Oncolytic Effect of Ad9xHRE1A against Renal Carcinoma Cells Is Mediated by HIF Activity. The experiments shown above demonstrate that Ad9xHRE1A replicates in VHL-deficient carcinoma cells but not in VHL-competent cells. Despite the best-characterized consequence of *VHL* loss is deregulation of HIF, it is very likely that other alterations also occur. To prove that Ad9xHRE1A replication in carcinoma cells is attributable to HIF activation and does not dependent on other factors, we generated a stable cell line expressing a mutant form of HIF-1α that is refractory to VHL regulation. Specifically, the proline residues 402 and 564 from human HIF-1α, required for VHL binding, were mutated to alanine and glycine residues, respectively, and this mutant construct was stably transfected into the VHL-competent WT-10 cell line to generate the WT-10-PP13 cell line. Fig. 6*A* shows that although both WT-10 and PP13 cells were efficiently eliminated by wild-type adenovirus, only WT-10 but not WT-10-PP13 showed resistance to Ad9xHRE1A infection. This result, together with the lack of effect of Ad9xHRE1A on VHL/HIF-defective UM-RC6 cells, indicate that HIF activity is necessary and sufficient to induce Ad9xHRE1A replication and killing.

These results also suggest that Ad9xHRE1A could be used to target VHL-competent cells under hypoxic conditions. This is of great interest because most solid tumors usually present large areas of hypoxic tissue, whereas HIF is not usually present in detectable amounts in normal tissues under physiological conditions (22–25). To test this possibility, we investigated the effect of Ad9xHRE1A on IMR-90 normal fibroblasts cultured under normoxic or hypoxic (1% oxygen) conditions. Unexpectedly, killing of IMR-90 cells by wild-type adenovirus was drastically reduced when cells were grown under hypoxic conditions (Fig. 6*B*), suggesting that, at least in this cell type, hypoxia interferes with the viral lytic cycle. This effect correlates with reduced expression of E1A mRNA as determined by Q-RT-PCR (data not shown). In contrast with the result obtained with the wild-type virus, Ad9xHRE1A kills IMR-90 cells more efficiently under hypoxic conditions (Fig. 6*B*). Considering the negative effect of hypoxia on the replication of wild-type adenovirus in this cell type, hypoxia enhances cell killing by Ad9xHRE1A very significantly.

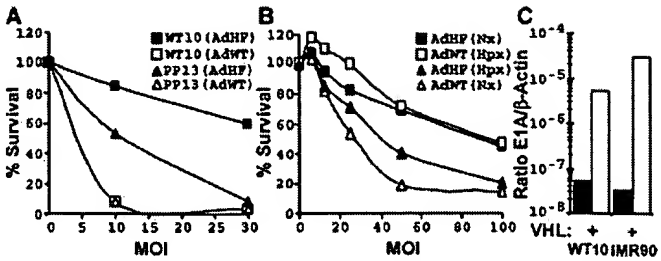

Fig. 6. Active HIF is sufficient to induce E1A expression and cytolysis by Ad9xHRE1A. WT-10 and WT-10-PP13 cell lines (*A*) or IMR90 fibroblast (*B*) were plated on 24-well plates, and 24 h later, they were infected with Ad9xHRE1A (*AdHF*) or wild-type (*AdWT*) adenovirus at the indicated MOIs. In the case of IMR90 cells, they were maintained in either hypoxic (1% oxygen, Hpx) or normoxic (Nx) conditions as indicated. Seven to 10 days after infections, the number of surviving cells was assessed by the MTT assay. The average values of duplicate determinations are represented as percentage of the value obtained for uninfected cultures. The experiments shown were repeated once with similar results. *C*, the human fibroblast IMR90 and the VHL-transfected renal carcinoma cells WT-10 were infected at a MOI of 100 pfu/cell with Ad9xHRE1A and then cultured for 12 h under normoxic (■) or hypoxic (□) conditions. The level of E1A expression was determined by Q-RT-PCR, and it is represented after normalization by the β-actin content.

In agreement with these results, hypoxia caused a strong induction (>100-fold) of E1A mRNA production in VHL-competent cells infected with Ad9xHRE1A (Fig. 6*C*). In conclusion, the absolute requirement of an active HIF for Ad9xHRE1A1A replication makes it suitable not only for the targeting of VHL-negative renal carcinomas but also for eliminating any other cell type growing under hypoxic conditions.

Oncolytic Effect of Ad9xHRE1A1A against RCCs *in Vivo*. The previous experiments demonstrate that Ad9xHRE1A is very efficient and specific in the elimination of cells with an active HIF transcription factors *in vitro* and suggest that it could be used to eliminate VHL-deficient tumors *in vivo*. To test this possibility, we generated VHL-deficient tumors in immunodeficient nude/nude mice by s.c. injection of 1-115 cells. When tumors reached and approximate volume of 200 mm^3, animals were treated by intratumor injection of Ad9xHRE1A or vehicle. Treatment was repeated once within 24 h of the first injection and then mice were left untreated for the rest of the experiment. As shown in Fig. 7, four of five tumors treated with Ad9xHRE1A showed a significant reduction of growth as compared with controls. Importantly, two of the four responsive tumors were completely eliminated by Ad9xHRE1A treatment (Fig. 7*A*). The behavior of the single unresponsive tumor can be explained by a nonefficient intratumor injection because its growth pattern clearly deviates from the rest of the treated group. To confirm the differences in growth of the two tumor groups, 60 days after initial treatment, animals were sacrificed and tumors excised to determine its weight (Fig. 7*B*). Unfortunately, we were unable to test the effect of Ad9xHRE1A against other VHL-deficient cell lines such as RCC10 *in vivo* because they produced small tumors that regressed spontaneously (data not shown).

Thus, Ad9xHRE1A is able to eliminate VHL-deficient cells not only *in vitro* but also *in vivo*. The effect of Ad9xHRE1A on 1-115 cells *in vivo* is of particular relevance given that this cell line is not very susceptible to adenoviral infection (Fig. 2).

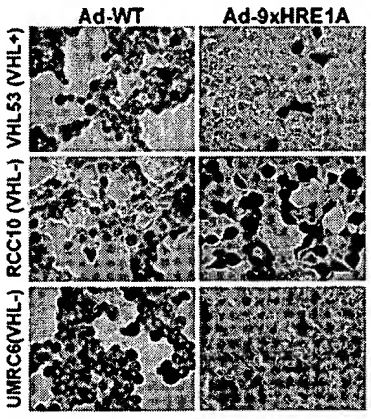

Fig. 5. The oncolytic effect of Ad9xHRE1A is due to viral replication. The indicated cell lines were infected with Ad9xHRE1A or wild-type virus at a MOI of 10 pfu/cell. After infections, cell cultures were extensively washed to eliminate any remaining infective particles in culture. Six to 7 days after infection, cell supernatants were collected and used as viral source to infect HEK293 cells. Forty-eight h after exposure to culture supernatants, HEK293 cells were fixed, and infected cells were visualized by immunostaining against the viral hexon protein. Representative microphotographs of HEK293 after immunostaining are shown. The experiment was repeated once with similar results.

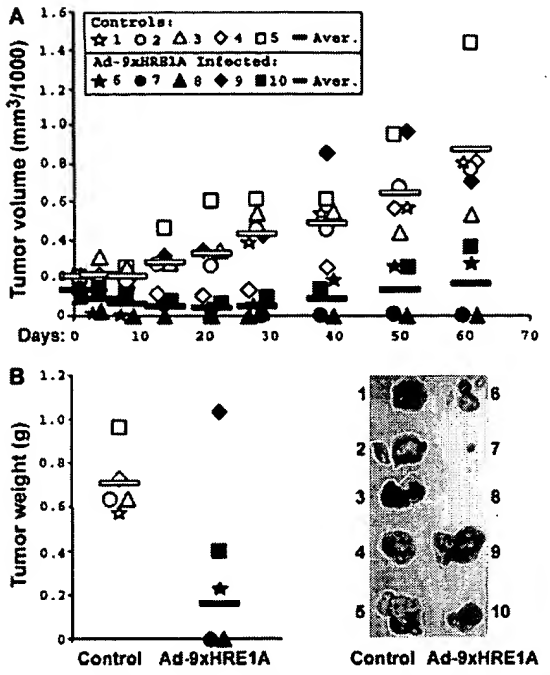

Fig. 7. *In vivo* antitumor effect of Ad9xHRE1A. *A*, a total of 10^7 1-115 (VHL-deficient) cells was s.c. injected into nude mice to generate accessible tumors. When tumors reached 200 mm^3, mice received intratumor injections of 1.6×10^8 pfu of Ad9xHRE1A (mice numbered 6–10) or vehicle alone (mice numbered 1–5) on 2 consecutive days. Then mice were left untreated for 60 days, and tumor growth was measured once/week. Each symbol represents the volume of a single tumor over time. *Open symbols* are used for the control-treated tumors, and *solid symbols* represent the tumors in the virus-treated group. The average volume for each animal group is shown (Aver., horizontal bar); animal number 9 was excluded to calculate mean of the virus-treated group. *B*, sixty days after initial treatment animals were sacrificed and tumors excised to determine its weight. The symbols represent each individual animal/tumor as in *A*. The average weight for each tumor group is shown (Aver., horizontal bar); animal number 9 was excluded to calculate the mean of the virus-treated group.

DISCUSSION

Here, we describe the generation and characterization of a new HIF-dependent conditionally replicative adenovirus for the treatment of VHL-deficient tumors. We have previously developed a conditionally replicative adenovirus to target breast tumors in which the E1A expression was driven by a dual estrogen and HIF-responsive promoter (7). However, this is the first description of an adenovirus in which the replication is controlled by HIF alone and the first study of a gene therapy approach to target clear cell renal carcinomas. In addition, the promoter described herein shows no (or extremely low) leaky expression, combined with high inducibility and strict dependence on HIF activity, as compared with the estrogen/hypoxia-inducible promoter previously reported (data not shown). All these characteristics make Ad9xHRE1A particularly well suited for the treatment of clear cell renal carcinomas defective for VHL function. It is of note that control of viral replication is achieved by the regulation of E1A gene expression from an artificial HIF-responsive minimal promoter instead of using the natural occurring promoter from a HIF-target gene. Naturally occurring promoters contain response elements for several different transcription factors, and as a consequence, they have limited value for the control of gene expression in response to a single specific alteration.

Loss or inactivation of *VHL* gene is responsible for the VHL cancer syndrome and the vast majority of sporadic clear cell renal carcinomas. One of the major problems affecting VHL patients is the development of multiple preneoplastic cysts in several organs, including liver, pancreas, and kidney. Another associated problem is the formation of hemangioblastomas because of the secretion of proangiogenic factors by the VHL-deficient stromal cells. It is likely that a therapy based on an agent such as Ad9xHRE1A could be potentially useful for the treatment of both cyst and hemanioblastomas in these patients. Because in these cases, the target of the therapy will be nonmalignant cells, no selection of cells resistant to therapy is expected. On the other hand, clear cell carcinomas of the kidney are discouragingly refractory to all chemotherapeutic regimens. Therefore, new therapeutic approaches against this disease are of great importance.

Although clear cell renal carcinomas are the most frequent kidney malignance, they comprise only the 2% of all human cancers. Importantly, however, human tumors other than renal carcinomas have been shown to overexpress HIF-α (23, 26, 27). In one study (23), a high proportion of the ovarian and colon carcinomas tested were positive for HIF-1α and HIF-2α inmunostaining. Despite the molecular mechanism responsible for HIF-α stabilization in these tumors is currently unknown, they are potential targets for Ad9xHRE1A. Finally, we found that hypoxia induced the replication of Ad9xHRE1A in cells with functional HIF regulatory pathways. Because almost all solid tumors present areas of hypoxic tissue where HIF-α protein is induced (28), the potential range of tumor targets for Ad9xHRE1A is wide. In agreement, a recent article (29) describes an HIF-regulated adenovirus for the targeting of hypoxic cells. In this vein, it is important to underline that despite some discrepancies (25), most authors agree that HIF-α protein is not expressed in the vast majority of the tissues under physiological conditions, with the exception of macrophages under some circumstances (22–24). Taking into account that expression of E1A by Ad9xHRE1 is strictly dependent on HIF activity, with very low or none leaky expression, this virus should be highly attenuated in normal cells.

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New Oncolytic Adenoviruses with Hypoxia- and Estrogen Receptor-Regulated Replication

RUBEN HERNANDEZ-ALCOCEBA, MICHAEL PIHALJA, DALONG QIAN, and MICHAEL F. CLARKE

ABSTRACT

Oncolytic adenoviruses with restricted replication can be produced if the expression of crucial transcription units of the virus is controlled by tissue- or tumor-specific promoters. Here we describe a method for the rapid incorporation of exogenous promoters into the E1A and E4 regions of the human adenovirus type 5 genome. Using this system, we have generated AdEHT2 and AdEHE2F, two conditionally replicative adenoviruses for the treatment of breast cancer. The expression of the E1A gene in both viruses is controlled by a minimal dual-specificity promoter that responds to estrogens and hypoxia. The tight regulation of E1A expression correlated with the ability of these viruses to replicate and kill human cancer cells that express estrogen receptors, or are maintained under hypoxic conditions. The telomerase reverse transcriptase (TERT) promoter and the E2F-1 promoter are preferentially activated in cancer cells. They were introduced into the E4 region of AdEHT2 and AdEHE2F, respectively. The telomerase core promoter failed to block the replication of the virus in telomerase-negative cells. In contrast, AdEHE2F was attenuated in nontransformed quiescent cells growing under normoxic conditions, suggesting that an intact pRB pathway with low levels of E2F transcription factors acts as a negative modulator for the virus. These data indicate that the simultaneous regulation of E1A and E4 viral transcription units by the appropriate combination of promoters can increase the tumor selectivity of oncolytic adenoviruses.

OVERVIEW SUMMARY

The lytic cycle of adenovirus leads to the death of the infected cells and the liberation of thousands of new viral particles. These properties can be exploited for the generation of therapeutic agents against cancer, if the replication of the virus is redirected to the malignant tissues. Three common characteristics found in most solid tumors are the existence of hypoxic areas, the expression of telomerase in the cells, and the elevation in free E2F transcription factors due to alterations in the pRB pathway. We have tested a combination of promoters that are activated under these circumstances in order to control the expression of regulatory proteins of adenovirus. We found that control of the viral E1A region by a hypoxia-responsive promoter and, to a lesser extent, control of the E4 region by an E2F-responsive promoter allow preferential replication of adenovirus in solid tumors.

INTRODUCTION

THE NEW GENERATION of conditionally replicative adenoviruses (CRADs) represents an advance over the previous replication-deficient vectors used in gene therapy against cancer. The ability to replicate their genome after infection allows them to amplify the effect that an initial load of vector may have. More importantly, they are therapeutic agents by themselves because they retain the ability to kill the cells as the last step of their lytic cycle (Alemany *et al.*, 2000). There are two major approaches to achieve preferential replication of adenovirus in cancer cells (Kruyt and Curiel, 2002). The so-called type 1 CRADs are based on the deletion or functional disruption of viral genes that activate the cell cycle in the infected cell, an effect that is needed for replication of the viral genome. This idea led to the development of *dl*1520, the first and most extensively used oncolytic adenovirus (Kirn, 2001; Biederer *et al.*, 2002). It contains a deletion in the E1B-55 kDa gene, which

Department of Internal Medicine, University of Michigan Comprehensive Cancer Center, Ann Arbor, MI 48109.

is essential for the virus to inactivate p53 and force the infected cell to enter the S phase of the cell cycle (Bischoff *et al.*, 1996). As a result, *dl*1520 shows preferential replication in those cells that have the p53 and related pathways already compromised by different mechanisms (Ries *et al.*, 2000; Yang *et al.*, 2001), as is the case in the majority of cancer cells. In accordance with the same principle, an adenovirus with a small deletion in the E1A gene targets cells with defects in the pRB pathway (Fueyo *et al.*, 2000; Heise *et al.*, 2000). However, these viruses are often attenuated even in the target cells, because the deletions can affect other functions of these viral genes. More recently, an alternative method to achieve tumor specificity has been described (Ramachandra *et al.*, 2001). The authors constructed a replication-competent adenovirus that expresses a repressor of E2F activity under the control of a promoter that responds to p53.

The second strategy to develop CRADs is based on the transcriptional control of genes that are necessary for replication of the virus. The viral genes are not modified; rather, they are placed under the control of promoters that are preferentially active in the tumor cells, compared with the surrounding tissues. E1A and E4 are the first two transcriptional units to be activated after infection, and both are required for efficient replication and viral production (Falgout and Ketner, 1987). The control of the E1A region is especially important because E1A acts as a strong activator for most of the transcription units in the adenovirus genome (Shenk, 1996). This means that if E1A is not expressed, not only is the virus unable to replicate, but also the expression of other viral proteins is greatly attenuated. The E4 region encodes a group of proteins that perform diverse functions and show a complex repertoire of interactions with other viral and cellular proteins (Tauber and Dobner, 2001). They are involved in replication of viral DNA, particle assembly, and interference with functions of the host cell such as competition for protein synthesis and induction of apoptosis (Halbert *et al.*, 1985; Branton and Roopchand, 2001). This latter effect can be important for an adequate release of virions once the viral DNA has been encapsidated. Therefore, controlling simultaneously the expression of E1A and E4 genes achieves a tighter control over replication of the virus (Hernandez-Alcoceba *et al.*, 2000; Fuerer and Iggo, 2002), and at the same time it decreases the risk of recombination and reversion to the wild-type genome. Promoters that have been used to control the E1A region include the prostate-specific promoter and enhancer for prostate cancer (Yu *et al.*, 1999), the osteocalcin promoter for bone metastases (Matsubara *et al.*, 2001), the midkine promoter for Ewing's sarcoma and neuroblastoma (Adachi *et al.*, 2001), the α-fetoprotein promoter for hepatocellular carcinomas (Hallenbeck *et al.*, 1999), the surfactant protein B (SPB) promoter for lung cancer (Doronin *et al.*, 2001), and promoters containing tcf/LEF transcription factor-binding sites for colon cancer (Fuerer and Iggo, 2002). These CRADs showed preferential replication in cancer cells, and demonstrated antitumor activity in preclinical models. However, for many human malignancies, including breast cancer, a tumor-specific promoter has not yet been characterized in detail. A CRAD utilizing the MUC-1 promoter in the E1A region has been made because this protein is overexpressed in many mammary tumors (Kurihara *et al.*, 2000), but some normal tissues also express this protein (Brugger *et al.*, 1999). In an initial approach to develop a CRAD for breast cancer, we constructed AdERE2 (Hernandez-Alcoceba *et al.*, 2000), a recombinant adenovirus that has both the E1A and E4 promoters deleted and substituted by a portion of the pS2 promoter containing two estrogen response elements (EREs). The estrogen receptor (ER) binds to these sequences and exerts its function as a transcriptional activator (Ekena *et al.*, 1998). This means that estrogens can activate the promoter only in cells that express ERs, which is the case in up to 70% of human breast cancers (Valavaara, 1997). In AdERE2, both E1A and E4 were efficiently controlled by this promoter, and we showed preferential replication and killing of breast cancer cells expressing ERs. In addition, AdERE2 was able to complement in *trans* the E1A deficiency of a replication-defective adenovirus vector expressing the proapoptotic gene encoding Bcl-xs.

One of the advantages of using transcriptional control for the generation of CRADs is the possibility of combining different response elements in the same regulatory unit to create artificial multispecificity promoters. In addition, different transcription units can be separately regulated in the same virus. In this way, the virus can be activated by a combination of stimuli that resemble the conditions present in tumor tissues. Because of the complexity of the adenovirus genome, the performance of these combinations of promoters can be tested only when the actual virus is produced. To facilitate this process, we have engineered a plasmid that contains the adenovirus genome with unique restriction sites flanking the E1A and E4 promoter regions. We have used this backbone to produce the CRADs that we describe here.

One common characteristic of most solid tumors is that they grow in an environment of low oxygen tension (hypoxia), due to the their aberrant vasculature (Zhong *et al.*, 1999; Talks *et al.*, 2000). Virtually all the cells in an organism have mechanisms for sensing and responding to lack of oxygen and nutrients. The transcription factor HIF (hypoxia-inducible factor) is an α/β heterodimer. The α subunit of this transcription factor is rapidly ubiquitinated and degraded by the proteasome under normoxic conditions, but the protein is stabilized in hypoxic environments (Huang *et al.*, 1998). The high levels of HIF induce the expression of a repertoire of genes necessary for adaptation to low oxygen. HIF binds to a sequence in the promoter of these genes, named the hypoxia responsive element (HRE). The use of this sequence as an enhancer for the expression of therapeutic genes has been proposed for the treatment of solid tumors (Dachs *et al.*, 1997). We have already described the characterization of a hybrid promoter containing HREs and EREs to control the expression of the proapoptotic gene *harakiri* (Hrk) (Hernandez-Alcoceba *et al.*, 2001). This therapeutic cassette was evaluated with a replication-deficient adenovirus, and we showed specific induction of apoptosis in ER^+ breast cancer cells, or in any other cell growing under hypoxic conditions. In the present study, we have used a minimal artificial promoter containing EREs and HREs to control the expression of E1A in two different CRADs, AdEHT2 and AdEHE2F. We placed the E4 region of these viruses under the control of two different promoters that have shown great potential for cancer targeting. In AdEHT2, this transcription unit is controlled by the core human telomerase reverse transcriptase (hTERT) promoter. Telomerase is a ribonucleotide complex composed of an RNA template (hTR) and a catalytic protein subunit with re-

verse transcriptase activity (hTERT) (Meyerson *et al.*, 1997). This enzyme is necessary to maintain the ends of the chromosomes (telomeres), which cannot be replicated by DNA polymerase. Telomerase adds the hexameric sequence TTAGGG, preventing the shortening and destabilization of the chromosomes after multiple rounds of cell division (Greider, 1996). Interestingly, telomerase activity is not detected in most human somatic cells, but, in contrast, the expression of hTERT is reactivated in most human cancers, as well as germ line cells and probably stem cells (Harle-Bachor and Boukamp, 1996; Ulaner *et al.*, 1998). The hTERT promoter has been characterized by different groups (Horikawa *et al.*, 1999; Takakura *et al.*, 1999; Wick *et al.*, 1999), and it has already been used to selectively direct the expression of proteins to cancer cells (Komata *et al.*, 2001; Majumdar *et al.*, 2001). Because both E1A and E4 are necessary for efficient viral replication, we hypothesized that having this promoter in the E4 region could add a second level of tumor specificity to the virus.

In the case of AdEHE2F, we placed the E4 region of the virus under the control of the E2F-1 promoter. E2F-1 belongs to a family of transcription factors that are functionally active in the G_1/S transition of the cell cycle. They bind to specific sites in the promoters of genes that are required for entry into the S phase (Polager *et al.*, 2002). Interestingly, the E2F-1 promoter itself contains E2F-binding sites (Johnson *et al.*, 1994). In quiescent cells, E2F factors are rendered inactive by binding to pRB and related proteins. In addition to this sequestration, the E2F–pRB complexes act as transcription repressors on the E2F sites (Sellers *et al.*, 1995). On the other hand, in transformed cells the pRB pathway is commonly altered, and therefore E2F activity is constantly elevated. Identification of the E2F-1 promoter (Hsiao *et al.*, 1994; Neuman *et al.*, 1994) offered the possibility of using it for transcriptional targeting of tumors (Parr *et al.*, 1997). It is important to mention that viral protein E1A can disrupt E2F–pRB complexes and therefore activate the E2F-1 promoter (Flint and Shenk, 1997). This means that the alteration in the pRB pathway might not be a requirement for replication of AdEHE2F if the promoter controlling the E1A region is already activated. However, in most normal tissues, the absence of ERs and the low levels of HIF and E2F activity should keep both E1A and E4 transcription units inactive (Fig. 1). In summary, we found both AdEHT2 and AdEHE2F viruses to be fully active in ER^+ cancer cells, or in cells growing under hypoxic conditions, in good correlation with the expression of the E1A region. Regarding the E4 region, we were unable to demonstrate restriction of AdEHT2 viral replication in telomerase-negative cells. On the other hand, we observed attenuation of AdEHE2F replication in normal quiescent cells, which suggests that the E2F-1 promoter retains its specificity in the context of the adenovirus genome.

MATERIALS AND METHODS

Cell lines

MCF7 (ATCC HTB 22) and BT-474 (ATTC HTB 20) are ER^+ human breast cancer cell lines. SK-BR-3 (ATCC HTB 30) is an ER^- human breast cancer cell line. Cells were maintained in RPMI medium (BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT). For MCF7 and BT-474, insulin (10 μg/ml; GIBCO-BRL, Grand Island, NY) was added. HeLa (ATCC CCL 2) is an ER^- human cervical cancer cell line, and it was maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL) supplemented with 10% FBS. IMR-90 (ATCC CCL 186) and BJ (ATCC CRL-2522) are human primary fibroblasts from lung and skin, respectively. They were maintained in Eagle's minimal essential medium (EMEM; BioWhittaker), supplemented with 10% FBS, 0.1 m*M* nonessential amino acids, and sodium

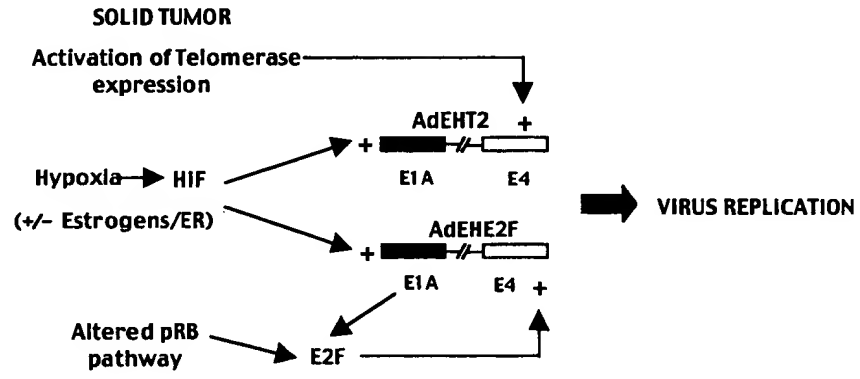

FIG. 1. Proposed mechanism for the preferential replication of AdEHT2 and AdEHE2F in solid tumors. On the left, some general characteristics of solid tumors are listed, and their influence on the replication of viruses is indicated with arrows. The E1A and E4 transcription units of adenovirus are represented as solid and open boxes, respectively. A hypoxic environment and the expression of estrogen receptors in the cells can independently activate the E1A unit of both viruses. The E4 unit of AdEHT2 is supposed to be activated in cells that express telomerase, and therefore it might attenuate the replication of the virus in telomerase-negative cells (see text). The E4 unit of AdEHE2F should be activated in cells that show alterations in the pRB pathway, because the levels of free E2F transcription factors are elevated in these cells. Because the viral E1A protein can produce the same effect, hypoxia and estrogens in ER^+ cells can indirectly activate the E4 unit of AdEHE2F. The expression of E1A and E4 units would allow adenoviral replication, destruction of the cells, and propagation of the infection within the tumor.

bicarbonate (1.5 g/liter) (GIBCO-BRL). NHFib cells are also human skin fibroblasts, and were maintained in DMEM supplemented with 10% FBS. Human dermal microvascular endothelial cells (HDMECs) were maintained in EBM-2 medium supplemented with EGM-2-MV (endothelial microvascular cell growth medium 2) additives (Clonetics, Walkersville, MD). NHK cells are normal human keratinocytes from skin. They were maintained in medium 154CF supplemented with HKGS (Cascade Biologics, Portland, OR). When the experiment required depletion of estrogens, IMEM without phenol red was used (GIBCO-BRL), supplemented with 2.5% charcoal–dextran-stripped serum (HyClone). Estradiol (17β-estradiol) and 4-OH-tamoxifen were purchased from Sigma (St. Louis, MO). A549 (ATCC CCL 185) is a human lung cancer cell line. It was maintained in Ham's F12 (BioWhittaker) supplemented with 10% FBS. 293E4pIX cells (Microbix, Toronto, ON, Canada) are a 293 cell line transformed with the adenovirus type 5 E4 transcription unit driven by the dexamethasone-inducible mouse mammary tumor virus (MMTV) promoter, and the protein IX unit driven by the $ZnCl_2$-inducible metallothionein promoter. It was maintained in EMEM supplemented with 10% heat-inactivated FBS. The expression of the E4 unit was induced by adding 1 μM dexamethasone (Gensia, Irvine, CA) to the culture medium. All culture media were supplemented with penicillin (100 U/ml), streptomycin (100 U/ml), and amphotericin B (Fungizone, 0.25 μg/ml; BioWhittaker).

Construction and characterization of reporter plasmids

The pB5XEH3 plasmid was constructed as follows. A cassette containing five copies of the ERE (GGTCACAGTGACC) plus a TATA box were subcloned into the polycloning site of the pGL2-Basic luciferase reporter plasmid (Promega, Madison, WI). Three copies of the HRE consensus element of the mouse phosphoglycerate kinase 1 (PGK-1) 5′ enhancer (TGTCACGTCCTGCACGAC) were then subcloned upstream of the ERE sequences, using the *Sma*I site. To verify the response to estrogens, the plasmid was transfected into MCF7 cells, using the FuGENE 6 reagent, as described by the manufacturer (Roche, Indianapolis, IN). The cells (typically 7.5×10^4/well in a 12-well plate) were incubated for 8 hr in estrogen-free medium and then transfected with 0.5 μg of the reporter plasmid and 0.1 μg of the plasmid pRLTK (Promega) in order to quantify the efficiency of transfection. Six hours later, the transfection medium was removed and new medium was added containing the indicated treatments. After 14–18 hr, cells were lysed and analyzed for luciferase activity, using the dual luciferase reporter assay system (Promega) as indicated by the manufacturer. The specific luciferase activity measured in a luminometer is presented as (firefly/*Renilla*) $\times$ 1000 luciferase units.

Construction of AdEHT2 and AdEHE2F

Using the plasmid pShutAd (Hernandez-Alcoceba *et al.*, 2000), we used standard molecular biology methods to produce a plasmid containing the E1A and E4 promoters flanked by unique restriction sites. The E1A promoter region (bp 341 to 499) was flanked by *Bst*BI sites. The E4 promoter region (bp 35619 to 35818) was flanked by I-*Ceu*I and *Swa*I sites, as shown in Fig. 2A. An insulator sequence (bovine growth hormone transcription stop signal) was subcloned upstream of the E1A promoter. The insulator was obtained from the pcDNA3 plasmid (Invitrogen, Carlsbad, CA) by digestion with *Pvu*II and *Not*I (Vassaux *et al.*, 1999). The core hTERT promoter (bp -208 to $+76$) and the E2F-1 promoter (bp -218 to $+51$) were amplified from genomic DNA with the Advantage-GC genomic PCR kit (Clontech, Palo Alto, CA). The primers used were 5′-TACTGTAACTATAACGGTCCTAAGGTAGCGACCAGTGGATTCGCGGGCACAG-3′ and 5′-TAAGTATTTAAATCGCGGGGGTGGCCGGGGCCAGGGCTTC-3′ for the hTERT promoter, and 5′-TACTGTAACTATAACGGTCCTAAGGTAGCGTGGTACCATCCGGACAAAGCC-3′ and 5′-TAAGTATTTAAATGGCGAGGGCTCGATCCCGC-3′ for the E2F-1 promoter. The plasmids containing the modified adenovirus genome were named pSEHT2 and pSEHE2F, respectively. For the production of viral particles, these plasmids were digested with *Pac*I, phenol–chloroform extracted and ethanol precipitated, and then transfected into 293E4pIX cells by the calcium phosphate method. The cells were treated with 1 μM dexamethasone until cytopathic effect (CPE) was observed. Individual plaques were isolated as described (Hernandez-Alcoceba *et al.*, 2000). Modifications in the E1A and E4 promoter regions were verified by polymerase chain reaction (PCR) using specific primers. The viruses were amplified in A549 cells, purified on CsCl gradients, and desalted in G-50 Sephadex columns. Titration was done according to the plaque-forming assay method. The titer was 1.1×10^7 and 0.7×10^7 PFU/μl for AdEHE2F and AdEHT2, respectively.

Analysis of transcriptional activation of E1A and E4 units

To analyze the responsiveness of the ERE/HRE promoter in the context of the adenoviral genome, we performed Northern blot assays of cells infected with AdEHT2 and AdEHE2F. Wild-type adenovirus type 5 (AdWT) was used as a control. Cells (5×10^6 cells/100-mm plate) were pretreated for 10 or 14 hr under the indicated conditions of estrogens and/or hypoxia. Infection was performed for 1 hr in 3 ml of culture medium with a multiplicity of infection (MOI) of 20 PFU/cell. The infection medium was then removed and the cells were incubated for 10 hr under the same conditions. Total RNA was extracted with TRIzol reagent (GIBCO-BRL), and 30 μg of RNA was formaldehyde–formamide denatured, fractionated in a 1.2% agarose gel, and transferred to a nylon membrane (Hybond-N+; Amersham, Buckinghamshire, UK). The E1A probe consisted of the 998-bp *Ssp*I–*Xba*I fragment from the adenoviral genome (nucleotides 341 to 1339), and the E4 probe was the 800-bp *Ssp*I–*Asn*I fragment (nucleotides 34634 to 35419). They were radiolabeled with a random primed DNA labeling kit (Roche). The membrane was prehybridized for 2 hr at 68°C in ExpressHyb hybridization solution (Clontech) in the presence of salmon sperm DNA (1 mg/ml) and tRNA (1 mg/ml). Hybridization was at 68°C for 1 hr with 2×10^6 cpm/ml of the probe. The membrane was then washed with 2× SSC, 0.1% sodium dodecyl sulfate (SDS) at room temperature for 20 min, and twice with 0.1× SSC, 0.1% SDS at 50°C (1× SSC is 0.15 *M* NaCl and 15 m*M* sodium citrate, pH 7.0). Finally, it was autoradiographed. To confirm homogeneous RNA loading and hy-

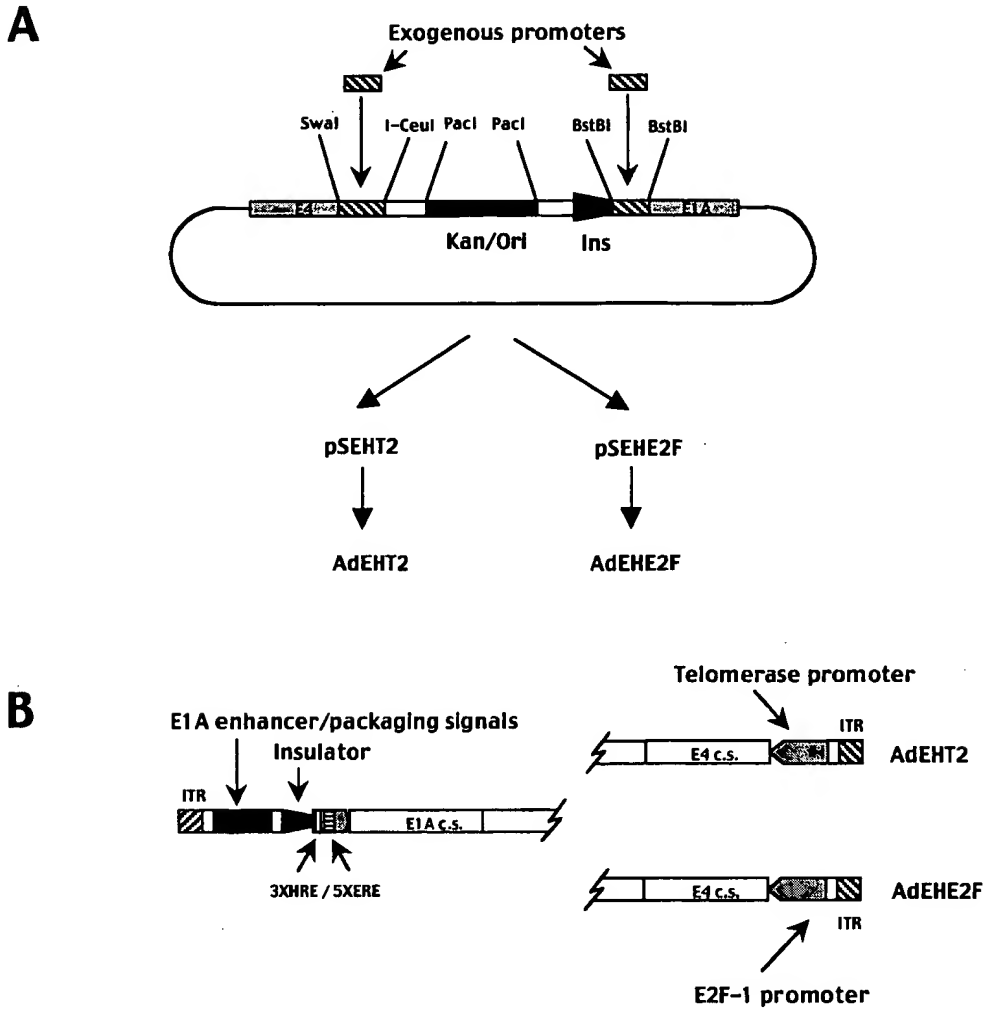

FIG. 2. Construction of AdEHT2 and AdEHE2F. (**A**) Method for the production of adenoviruses with modifications in the E1A and E4 promoters. These regions were flanked by unique restriction sites (*Bst*BI for E1A and *SwaI*/I-*CeuI* for E4) in a plasmid that contains the entire adenovirus type 5 genome. After subcloning the exogenous promoters, the plasmids obtained (pSEHT2 and pSEHE2F in this case) are digested with *Pac*I in order to liberate the viral genome, and they are transfected in permissive cells to generate the CRADs AdEHT2 and AdEHE2F, respectively. Kan, Kanamycin resistance; Ori, origin of replication for *E. coli*; Ins, insulator sequence. (**B**) Schematic representation of the modifications introduced in the viral genome. An insulator sequence was placed between the overlapping E1A enhancer/packaging signals and the E1A promoter. The wild-type E1A promoter was substituted with an artificial minimal promoter containing three hypoxia responsive elements (HREs) and five estrogen response elements (EREs). The E4 promoter was substituted with the hTERT and E2F-1 promoters in AdEHT2 and AdEHE2F, respectively. ITR, Inverted terminal repeats; c.s., coding sequence.

bridization, we hybridized the same membranes with a human β-actin probe.

Cytopathic effect and viability of cells

After infection of cells with the different viruses, the appearance of cytopathic effect (rounding and detachment) was monitored and cells were photographed with a digital camera (Pixera, Los Gatos, CA), typically 9 days after infection (data not shown). To quantify the viability of cells, the MTT assay was used (Sigma). At the time of analysis, cells were washed with 1 ml of phosphate-buffered saline (PBS) and 300 μl of a solution containing thiazolyl blue (MTT) solution (0.8 mg/ml) was added to the cells. Five hours later, 300 μl of solubilization solution (20% [w/v] SDS in 50% [v/v] *N,N*-dimethylformamide) were added, and 12 hr later the absorbance at 600 nm was read in a spectrophotometer. The significance of the differences observed was analyzed by *t* test.

In vivo *assays in nude mice*

The antitumor effect of AdEHT2 and AdEHE2F was tested in human tumor xenografts implanted in 5- to 7-week-old Hsd *nu/nu* mice (Harlan, Indianapolis, IN). Estradiol pellets (0.72 mg of 17β-estradiol, 60-day release; Innovative Research of America, Sarasota, FL) were implanted subcutaneously in the

mice. Two days later, 15×10^6 BT-474 cells were resuspended in serum-free RPMI plus 80% Matrigel (Collaborative Biomedical Products, Bedford, MA) and injected subcutaneously into the second left breast. When tumors reached an average volume of approximately 200 mm^3, viruses were diluted to 5×10^8 PFU in 100 μl of PBS and injected intratumorally. Tumor volume was measured weekly and calculated according to the equation $(D \times d^2)/2$, where D and d are the major and minor diameters, respectively. The significance of the differences observed was analyzed by t test.

The toxicity assays were performed in Hsd *nu/+* mice by intravenous injection of 100-μl virus solutions in PBS via the retro-orbital plexus. Mice were monitored daily for signs of mortality and morbidity. Two days after viral injection, blood samples were collected and levels of the hepatic enzyme alanine aminotransferase (ALT) were analyzed in order to assess liver toxicity.

RESULTS

Construction of AdEHT2 and AdEHE2F

To test different combinations of promoters in the E1A and E4 transcription units of adenovirus, we constructed a plasmid containing the adenovirus type 5 genome with these regions flanked by unique restriction sites (Fig. 2A), as suggested by Mizuguchi and Kay (1998). In the E4 region, we subcloned the hTERT promoter (virus AdEHT2) or the E2F-1 promoter (virus AdEHE2F), as indicated in Fig. 2B. These promoters have already been described by other groups (Hsiao *et al.*, 1994; Neuman *et al.*, 1994; Horikawa *et al.*, 1999; Takakura *et al.*, 1999; Wick *et al.*, 1999). The control of the E1A region is crucial for the regulation of CRADs. The wild-type virus contains a strong enhancer for E1A that overlaps with the packaging signal. To prevent any possible interference with the function of the exogenous promoter used in this area, we introduced an insulator sequence (bovine growth hormone transcription stop signal) between this enhancer and the location of the E1A promoter. In an effort to minimize the basal activity of the promoter, we constructed and characterized a new minimal promoter containing three HREs and five EREs plus a TATA box. A luciferase reporter was used to test its activation by estrogens and hypoxia (Fig. 3). The ER^+ human breast cancer cell line MCF7 was transiently transfected with this plasmid and the cells were exposed to estrogens (2.5 nM 17β-estradiol), hypoxia (1% O_2), or both stimuli together. The result shows that this promoter has low basal activity in the absence of estrogens (Fig. 3A). As described previously for a similar promoter, both estrogens and hypoxia stimulated transcription, which means that the HRE and ERE sites can work independently. However, in the case of this artificial promoter the response to estrogens (25-fold over the untreated cells) was lower than that obtained with hypoxia (80-fold). The addition of the estrogenic competitive inhibitor 4-OH-tamoxifen partially blocked the response to estrogens, indicating that the induction is specific (Fig. 3A). In ER- HeLa cells, no elevation of the luciferase activity was observed, whereas hypoxia was still able to stimulate transcription (Fig. 3B). In summary, the ERE/HRE minimal promoter combined low basal activity with good inducibility. Its reduced size and the lack of potentially undesirable response elements make it a good candidate for substitution of adenoviral regulatory sequences. We then introduced it in the adenovirus genome, replacing the wild-type E1A promoter in both AdEHT2 and AdEHE2F (Fig. 2B).

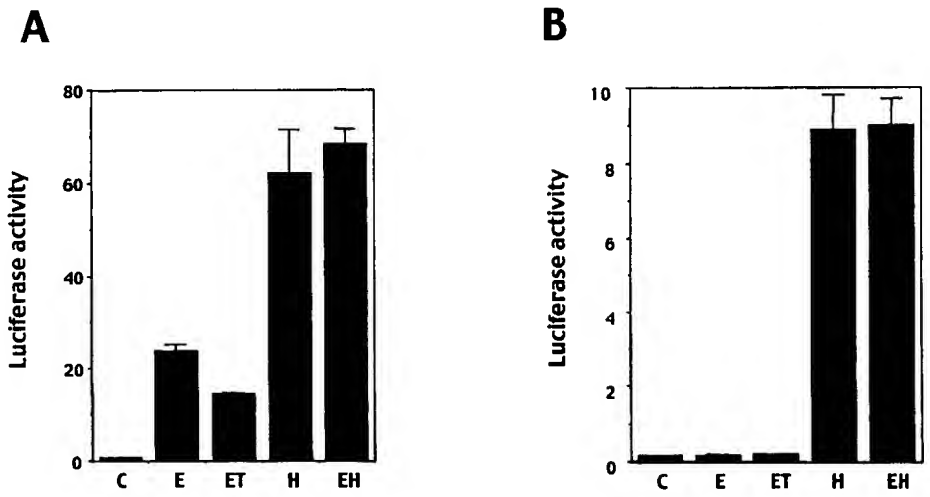

FIG. 3. Characterization of the ERE/HRE minimal promoter. The pB5XEH3 luciferase reporter plasmid containing this promoter was transfected into MCF7 cells (**A**) or HeLa cells (**B**) that had been estrogen deprived for 8 hr. The constitutive *Renilla* luciferase reporter pRLTK was cotransfected in order to control the efficiency of transfection. Six hours later, the transfection medium was removed and new medium containing the indicated treatments was added. After 16 hr, the cells were lysed and analyzed for luciferase activity. The columns represent the equation (firefly/*Renilla*) luciferase units $\times 10^3$. Note that the y axis range is different in each panel. C, Untreated; E, 2.5 nM 17β-estradiol; ET, 2.5 nM 17β-estradiol plus 2.5 μM 4-OH-tamoxifen; H, hypoxia (1% O_2); EH, 2.5 nM 17β-estradiol plus hypoxia. The experiment was repeated five times for MCF7 cells and two times for HeLa cells, with similar results.

The viral particles were produced by transfection of the respective plasmids, as described in Materials and Methods.

Transcriptional control of the E1A and E4 regions

Once the AdEHT2 and AdEHE2F viruses were produced, we investigated whether the exogenous promoters were able to control the expression of E1A and E4 in the context of the adenovirus genome. We used MCF7 as an example of an ER^+ cell line and HeLa as an example of an ER^- cell line. IMR-90 cells are nontransformed human primary fibroblasts from lung that suffer senescence after a series of passages in culture. We verified by reverse transcriptase (RT)-PCR that MCF-7 and HeLa cells express telomerase, whereas IMR-90 cells are negative (data not shown), as described in the literature (Meyerson *et al.*, 1997; Majumdar *et al.*, 2001). Cells were infected for 10 hr with an MOI of 20 PFU/cell of either AdEHT2, AdEHE2F, or AdWT as a control. Cells were maintained in RPMI medium supplemented with 2% FBS and 2 n*M* 17β-estradiol, with or without hypoxia (1% O_2). In addition, a set of MCF7 cells was incubated in the absence of estrogens. Total RNA was extracted and expression of the E1A and E4 transcription units was analyzed by Northern blot using specific probes, as shown in Fig. 4. There is a tight regulation of the E1A region in both AdEHT2 and AdEHE2F. In all the cell lines tested, hypoxia induced strong expression of E1A. Importantly, the expression was undetectable in the ER^- cell line HeLa under normoxic conditions. In MCF7 cells basal expression was low, estrogens caused activation, and the best response was obtained by hypoxia and estrogens together. These data indicate that the minimal ERE/HRE promoter maintains its properties of low basal activity and high inducibility in the context of the adenoviral genome. The use of an insulator sequence might contribute to the tight regulation of the E1A region in these viruses. On occasion, cells infected with AdWT showed a slight increase in E1A expression in response to hypoxia, as is the case for HeLa in this assay. In fact, analysis of the left arm of the adenovirus genome reveals some HRE-like sequences. The significance of this finding is unclear, because the ability of the wild-type virus to replicate was not significantly increased by hypoxia (see below). Probably the increase in E1A expression above a certain threshold is irrelevant for its function. In contrast with the high levels of E1A obtained in cells infected with the recombinant adenovirus, the expression of the E4 region in both viruses was relatively low when compared with the wild-type virus. This is especially true for IMR-90 cells, which lack expression of telomerase. However, it is not clear that this is due to specific regulation of the hTERT or E2F-1 promoter. As can be seen in HeLa cells infected for 10 hr with AdEHT2 and AdEHE2F, the expression of E4 is low unless the cells are maintained under hypoxic conditions. This suggests that the signals controlling expression of telomerase or the basal levels of E2F in these

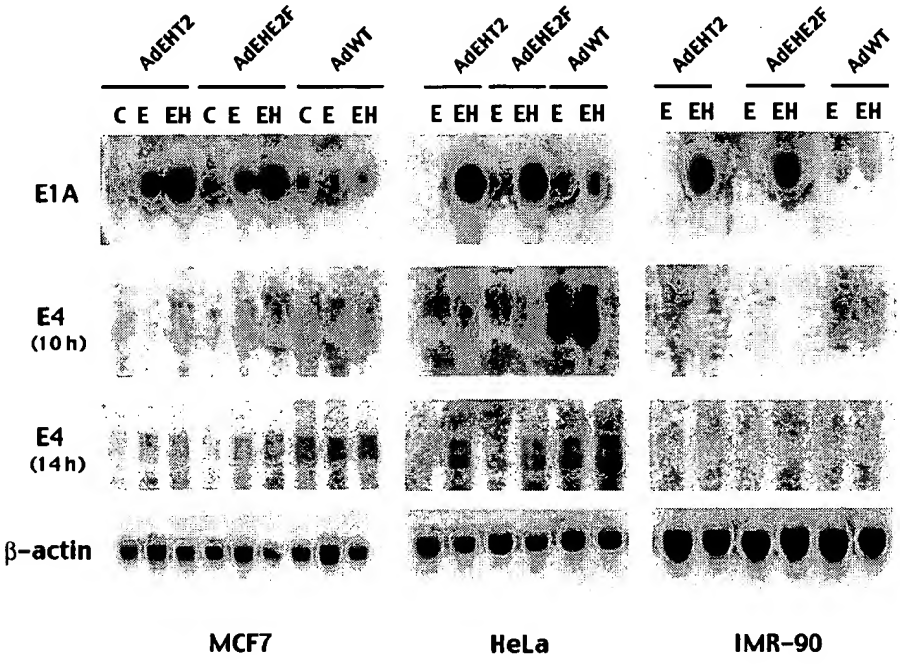

FIG. 4. Transcriptional control of the E1A and E4 units. MCF7, HeLa, and IMR-90 cells were pretreated for 10 hr with 2 n*M* 17β-estradiol (E) or 2 n*M* 17β-estradiol plus hypoxia (EH). In addition, one set of MCF7 cells was maintained in estrogen-depleted medium (C). Cells were then infected with an MOI of 20 PFU/cell of AdEHT2, AdEHE2F, or AdWT, under the same treatment conditions. Ten or 14 hr after infection, total RNA was extracted. Northern blot analysis was performed with radioactive probes specific for the E1A region, E4 region, or the constitutively expressed β-actin gene as a control. The intensity of the signal should not be compared between different cells because in this short period of time, it reflects mostly the sensitivity of the cells to adenovirus infection (which is highest in HeLa cells and lowest in IMR-90 cells, as can be deduced from the intensity of the signal obtained in AdWT-infected cells). The times of exposure of the autoradiograms are different for each blot. The experiment was repeated three times with similar results.

transformed cells are by themselves unable to stimulate E4. However, under hypoxic conditions the levels of E1A are high and this appears to cooperate in the activation of the E4 promoter. In the case of AdEHE2F, this may be a consequence of the sequestration of pRB by E1A. In AdEHT2, a less specific mechanism could be taking place, because E1A is known as a ubiquitous activator of transcription in many promoters (Flint and Shenk, 1997), and that may be happening to the hTERT promoter as well. To evaluate the regulation of the E4 region in more detail, we repeated the Northern blot assays 14 hr after infection, as indicated in Fig. 4. At this time, the influence of E1A is more evident. In fact, the expression of E4 in MCF7 and HeLa cells infected with the CRADs clearly responds to the same stimuli (expression of ERs and hypoxia). Although E4 is still low in IMR-90 cells, a slight increase can be observed under hypoxic conditions, especially in the case of AdEHE2F. In summary, the activity of the E4 unit driven by the hTERT and E2F-1 promoters is attenuated in normal cells, but it can be influenced by the levels of E1A.

Modulation of the cytotoxicity of AdEHT2 and AdEHE2F by hypoxia

Hypoxia is a strong activator of the E1A region for both AdEHT2 and AdEHE2F, and this also affects expression of the E4 region indirectly. We then examined the consequences of this regulation on the ability of these viruses to replicate and kill cells in culture. We compared the ER^+ cell lines MCF7 and BT-474, and the ER^- cell lines HeLa and SK-BR-3. Cells were infected with AdEHT2, AdEHE2F, or AdWT at an MOI of 10 PFU/cell and viability was quantified 9 days after infection, using the MTT assay (Fig. 5). We chose an MOI that caused no CPE in the first 3 days postinfection even with the wild-type virus, to make sure that any effect that appears later on is due to virus replication. All the cells were incubated in the presence of 17β-estradiol; some of them under normoxic and some under hypoxic conditions. In good correlation with the expression of E1A, both recombinant adenoviruses caused extensive death of MCF7 cells in the presence of estrogens (90% decrease in viability), with or without hypoxia. The same was true for the other ER^+ cell line BT-474, with virtually complete eradication of cells at the end of the experiment. On the other hand, HeLa cells were not significantly affected under normoxic conditions, which suggests that the expression of ER is necessary for activation of the viruses in the absence of hypoxia. However, lowering the oxygen tension to 1% caused significant activation of the capacity of the CRADs to kill these cells, with 80% reduction in viability observed with AdEHE2F. In the case of SK-BR-3, hypoxia stimulates the cytotoxicity of the recombinant adenoviruses, but the attenuation under normoxic conditions is only partial, especially for AdEHE2F. Interestingly, this cell line overexpresses the *Her2/neu* oncogene (Moulder *et al.*, 2001), which can cause increased levels of HIF in the absence of hypoxia (Laughner *et al.*, 2001). Note that the ability of AdWT to kill cells was not affected by hypoxia. In summary, hypoxia induced a significant increase in cytotoxicity only for AdEHT2 and AdEHE2F, and the expression of ERs in the cells had a permissive role for these viruses. Finally, we performed a dose–response analysis and calculated the MOI of virus necessary to kill 50% of the cells in order to estimate the extent of

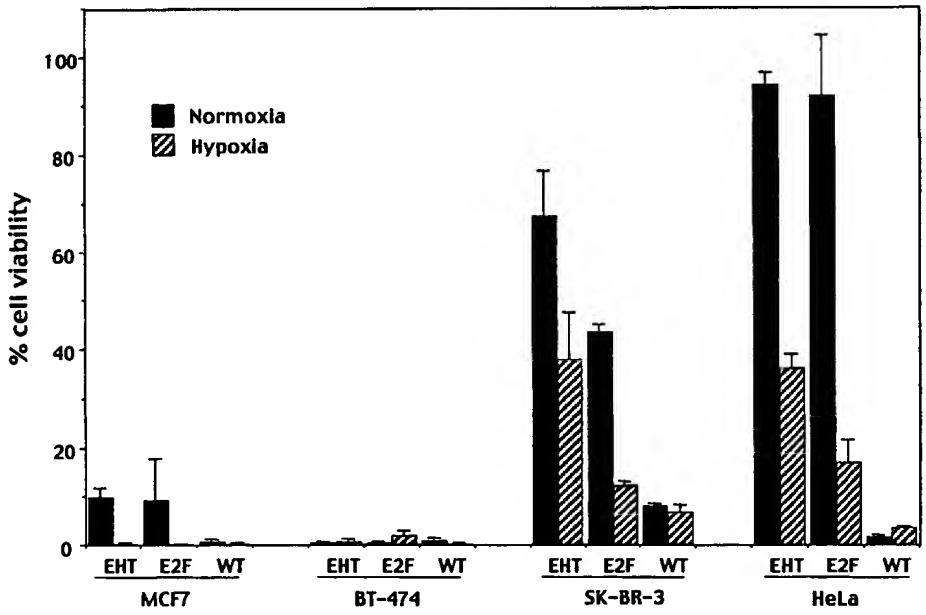

FIG. 5. Viability of different human cancer cell lines infected with AdEHT2 and AdEHE2F. The ER^+ cell lines MCF7 and BT-474, and the ER^- cell lines HeLa and SK-BR-3, were infected with AdEHT2 (EHT), AdEHE2F (E2F), or AdWT (WT) at an MOI of 10 PFU/cell in the presence of 2 n*M* 17β-estradiol. Cells were maintained under normoxic conditions (solid columns) or hypoxic conditions (hatched columns) for 9 days, and viability was analyzed by the MTT assay. The columns represent the percentage of surviving cells, compared with uninfected cells growing under the same conditions. The experiment was repeated five times with similar results.

TABLE 1. MODULATION OF CYTOTOXICITY OF AdEHT2 AND AdEHE2F BY HYPOXIA[a]

	AdEHT2		*AdEHE2F*		*AdWT*	
	N	*H*	*N*	*H*	*N*	*H*
BT-474	2	0.5	0.4	0.2	0.05	0.07
HeLa	>100	19	60	6	1	1

[a]BT-474 and HeLa cells were infected with the indicated viruses at different MOIs in the presence of 2 n*M* 17β-estradiol, under normoxic (N) or hypoxic (H) conditions. Ten days after infection, the viability of the cells was evaluated by the MTT assay. Shown are the MOIs that caused 50% loss of viability compared with uninfected cells.

regulation by hypoxia. In HeLa cells hypoxia can stimulate at least 10-fold the ability of CRADs to eliminate these cells (Table 1). In BT-474 cells, the maximum cytotoxicity effect was always achieved when both estrogens and hypoxia were present, but the expression of ERs allowed nearly full activity of the viruses. Overall, AdEHE2F was 3-fold more cytotoxic than AdEHT2, but it was still attenuated about 5-fold when compared with the wild-type adenovirus, whose cytotoxicity was not affected by hypoxia. In summary, these results indicate that the expression of ERs in the cells, or the existence of a hypoxic environment, are key factors that control the proliferation and cytotoxicity of the new CRADs.

Evaluation of the hTERT and E2F-1 promoters in the E4 region of adenovirus

The purpose of placing the E4 region of adenovirus under the control of the hTERT promoter was to confine the replication of adenovirus only to cells that express telomerase. Northern blot assays showed that the activity of the E4 region of AdEHT2 seemed to be attenuated in IMR-90 primary cells, which do not express this enzyme (Fig. 4). However, the activation of the promoter in HeLa or MCF7 cells was also low, and it was apparent only when E1A expression was also activated by hypoxia. Despite the low levels of E4 in MCF7 cells under normoxic conditions, AdEHT2 was still able to replicate and kill these cells. The consequences of this situation in terms of virus replication and cytotoxicity were evaluated in a viability assay using telomerase-negative IMR-90 cells. As shown in Fig. 6A, when these cells were infected under hypoxic conditions, AdEHT2 was not attenuated. In fact, it showed effects similar to those of the wild-type virus, suggesting that the influence of E1A expression activated by hypoxia is sufficient to allow replication of the virus. When the same experiment was repeated with AdEHE2F, a more pronounced reduction of cell viability was observed with hypoxia treatment (Fig. 6B). In this case the result is not surprising if we consider that in some situations, E1A can activate the E2F-1 promoter by blocking the pRB pathway (Flint and Shenk, 1997).

To evaluate the ability of the E2F-1 promoter to attenuate the replication of adenovirus in quiescent cells, we compared the viability of IMR-90 cells infected with AdEHE2F in serum-free medium versus medium that contains 2% FBS. Unlike most cancer cell lines, these primary cells do not express E2F-1 and other proliferation-related transcription factors when they are maintained in serum-free medium, but serum can reactivate their expression (Good *et al.*, 1996). Figure 7A shows that, as expected, AdEHE2F was attenuated in quiescent cells. This effect was not a general characteristic of adenovirus, because it was not observed with AdWT. Actually, the cells were slightly more sensitive when infected with this virus in serum-free medium (Fig. 7B). In addition, the attenuation did not take place in the breast cancer cell line MCF7 (Fig. 7C). These results suggest that the E2F-1 promoter controlling the E4 region can help to diminish the replication of adenovirus in normal somatic

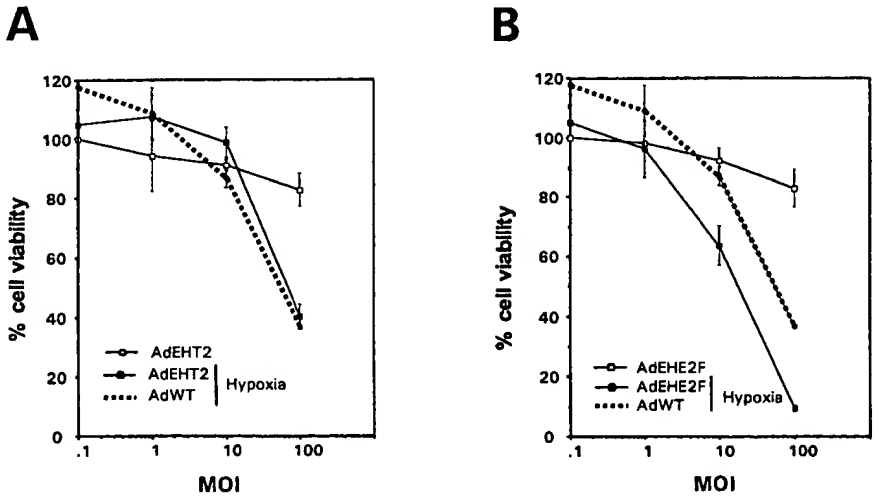

FIG. 6. Viability of IMR-90 cells infected with AdEHT2 and AdEHE2F. Telomerase-negative IMR-90 cells were infected with AdEHT2 (**A**) or AdEHE2F (**B**) at different MOIs in the presence of 2 n*M* 17β-estradiol, under normoxic (open squares) or hypoxic (solid squares) conditions. As a control, cells were infected with AdWT under hypoxic conditions (discontinuous line). The viability of the cells was analyzed 10 days after infection, using the MTT assay. The percentage of viable cells is presented on the *y* axis and the MOI in logarithmic scale is presented on the *x* axis. The experiment was repeated three times with similar results.

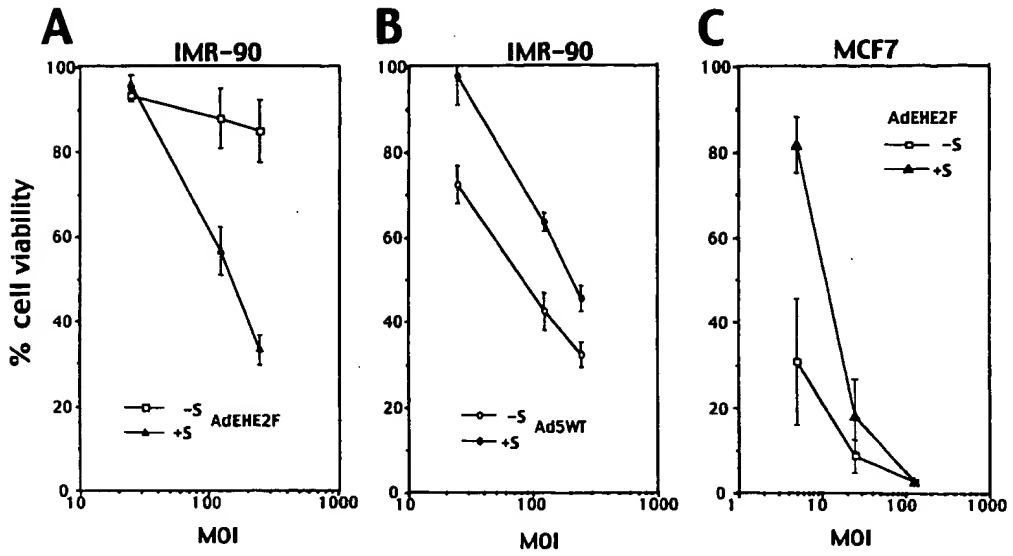

FIG. 7. Cytotoxicity of AdEHE2F in quiescent cells. (A) IMR-90 cells were infected with different MOIs of AdEHE2F in the presence of 2 n*M* 17β-estradiol. The cells were maintained in serum-free medium (open squares), or in the usual infection medium containing 2% FBS (solid triangles). The viability was analyzed 8 days after infection, using the MTT assay. The percentage of viable cells is presented on the *y* axis and the MOI in logarithmic scale is presented on the *x* axis. The same experiment was performed with the AdWT virus and IMR-90 cells (B), and with AdEHE2F virus and MCF7 cells (C). Note that the MOIs used are lower in the case of MCF7 cells. For this assay, we infected a higher initial number of IMR-90 cells than in previous viability assays (2×10^4 versus 5×10^3), because these cells do not proliferate without serum and at the end of the incubation period they would fall below the detection limit of the MTT assay. Therefore, (A) and (B) cannot be compared quantitatively with (C), because the same MOIs actually mean more plaque-forming units per volume of infection medium, and this can affect the efficiency of infection. The experiment was repeated three times with similar results.

cells. To validate this point, we tested four additional nontransformed human cells: BJ and NHFib are fibroblasts derived from skin, HDMECs are endothelial cells, and NHK cells are keratinocytes derived from skin. The cells were infected under normoxic conditions with either AdEHE2F or AdWT at different MOIs. The viability of the cells was analyzed when the monolayers infected with AdWT showed extensive cytopathic effect for each cell type. As shown in Fig. 8, the normal cell populations maintained at least 70% viability when infected with AdEHE2F, whereas the same amount of AdWT killed virtually all the cells. However, when we performed AdEHE2F infection under hypoxic conditions, the ability of the virus to replicate and kill these cells was greatly activated, reaching levels similar to the wild-type version. This suggests that the attenuation of AdEHE2F in normal cells is specific, and is not simply due to a general reduction of its ability to induce cell death compared with AdWT.

Toxicity of AdEHE2F in mice

To evaluate whether the specificity observed with AdEHE2F in cultured cells correlated with a reduction in toxicity *in vivo*, we performed intravenous injections of either AdEHE2F, AdWT, or the E1-deleted adenoviral vector AdΔE1. Increasing amounts of viral particles were injected into the retro-orbital plexus of Hsd *nu/+* mice. We monitored mortality, weight loss, and damage to liver by quantification of the enzyme alanine aminotransferase (ALT). The results are summarized in Fig. 9. We observed that any dose above 1×10^{11} particles of AdWT causes death of all the mice 4 days after injection, with severe elevation of ALT levels in the first 48 hr. A dose of 0.6×10^{11} particles induces a moderate increase in ALT, but no deaths or weight loss occurred. On the other hand, AdEHE2F was well tolerated at 1×10^{11} particles, with no mortality and no liver toxicity. This virus was lethal to most of the mice at doses above 4.5×10^{11} particles, whereas 2.2×10^{11} particles caused the death of only one of four animals. Interestingly, ALT levels in mice that died after AdEHE2F administration were generally lower than those observed for AdWT. In fact, most of the mice died with enzyme levels only twice the upper normal limit (about 120 U/L). Finally, AdΔE1 showed no signs of toxicity even at the highest dose tested (4.5×10^{11} particles). Only a slight increase in ALT levels, still within the physiological range, could be observed. In summary, the toxicity of AdEHE2F is reduced compared with the wild-type virus, but it is somewhat more toxic than a replication-deficient adenoviral vector.

Antitumor effect in vivo

We evaluated the ability of AdEHT2 and AdEHE2F to inhibit the growth of human breast cancer xenografts established in estrogen-treated nude mice. The tumors were generated by orthotopic injection of BT-474 cells into the mammary fat pad. When they reached an average of 200 mm^3, 5×10^8 PFU of virus was injected intratumorally, three consecutive days per week for a total of four weeks. No side effects related to virus administration were observed. Variation in the tumor volume of treated and control mice is shown in Fig. 10. Both AdEHT2

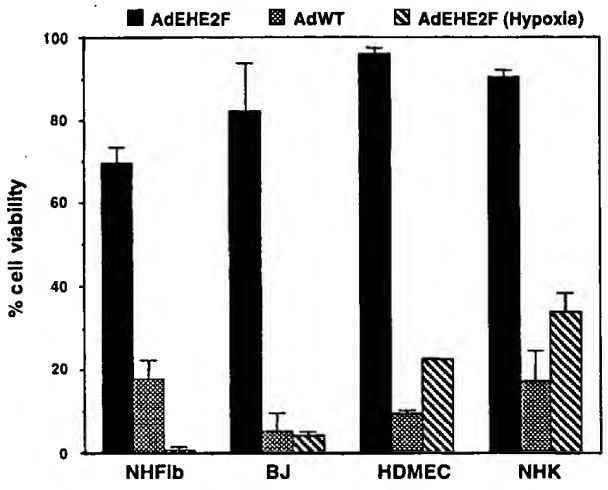

FIG. 8. Cytotoxicity of AdEHE2F in human nontransformed primary cells. BJ and NHFib skin fibroblasts, NHK keratinocytes, and human dermal microvascular endothelial cells (HDMECs) were infected with AdEHE2F (solid columns) and AdWT (cross-hatched columns) with an MOI of 750 PFU/cell for LEHFib, 500 PFU/cell for BJ, and 10 PFU/cell for HDMECs and NHK cells. In addition, the cells were infected with the same amount of AdEHE2F and maintained under hypoxic conditions (hatched columns). Viability was analyzed by MTT assay, 16 days after infection for BJ cells and 9 days after infection for LEHFib cells, HDMECs, and NHK cells. The columns represent the percentage of surviving cells, compared with uninfected cells growing under the same conditions. The experiment was repeated three times with similar results

and AdEHE2F showed similar antitumor effects. The reduction in tumor size compared with the control group was significant after the second week of treatment ($p < 0.05$), and was close to 90% 2 weeks later. Both viruses completely inhibited the growth of tumors. The size of untreated tumors increased more than 4-fold before they reached a plateau, whereas the treated tumors remained smaller than their original size. However, residual lesions could be persistently detected in most of the treated animals.

DISCUSSION

The ability of CRADs to complete the lytic cycle of adenovirus makes them a promising platform to develop new viral or gene therapy strategies against cancer. However, every step of this cycle is subjected to complex regulation that is still not fully understood. The degree and time of expression of viral proteins, as well as their repertoire of interactions with cellular counterparts, need to be compatible with productive infection. When E1A and E1B genes of adenovirus are mutated or deleted to make the replication of the virus tumor specific (type 1 CRADs), its performance can decrease because some basic functions of the proteins can be affected. On the other hand, when the promoters for the E1A and E4 genes are replaced by tumor or tissue-specific promoters (type 2 CRADs), it is difficult to predict what impact this will have on replication of the virus. The combination of promoters used in this study can provide some information about the latter question. Our results support the notion that a tight regulation of the E1A region is critical for the control of adenovirus replication. This can be achieved by using an artificial, minimal promoter that contains only the response elements required for its function, in this case the EREs and HREs. This minimizes the possibility that the promoter can be activated in certain undesirable cell types or situations that are difficult to test by transient transfections with reporter plasmids. Both AdEHT2 and AdEHE2F showed a good activation of E1A expression by hypoxia in different cell lines, or by estrogens in ER^+ cells. This correlated well with increased cytotoxicity under the same circumstances. In solid tumors, the activation of viral replication in hypoxic areas can create multiple sites in which the concentration of the virus is high. This could cause destruction of surrounding areas of the tumor, even if they are not hypoxic, until the titers of virus decrease radially into the normoxic areas. This could happen independently of the ER status of the tumor. However, in the case of an ER^+ tumor, like many breast cancers, replication of these CRADs would be activated beyond the hypoxic areas. Again, this may not require that every single cell in the tumor be ER^+. In addition, the use of a promoter activated by HIF can provide tumor specificity by itself, because overexpression of this transcription factor may be associated with the transformed phenotype of some cancer cells (Bos *et al.*, 2001).

For several reasons, analyzing the function of the promoters used in the E4 region of these viruses is more complex. First, both of them contain binding sites for transcription factors

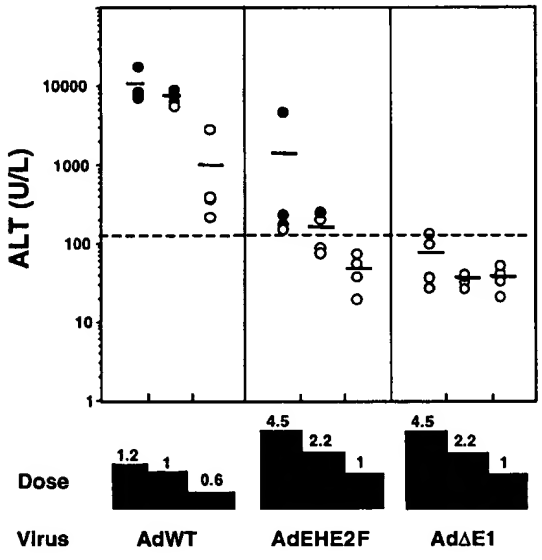

FIG. 9. Toxicity of AdEHE2F. Mice ($n = 4$ per group) were injected intravenously with increasing amounts of either AdWT (*left*), AdEHE2F (*center*), or AdΔE1 (*right*). The doses ($\times 10^{11}$ particles) are indicated below each group. Shown are the serum levels of alanine aminotransferase (ALT) in units per liter for every animal, analyzed 48 hr after infection. The solid columns indicate the mean value for each group. Open circles represent mice that survived 10 days after infection, whereas solid circles indicate mice that died, usually 4 days after infection. The discontinuous line indicates the maximum normal value for ALT (approximately 120 U/L).

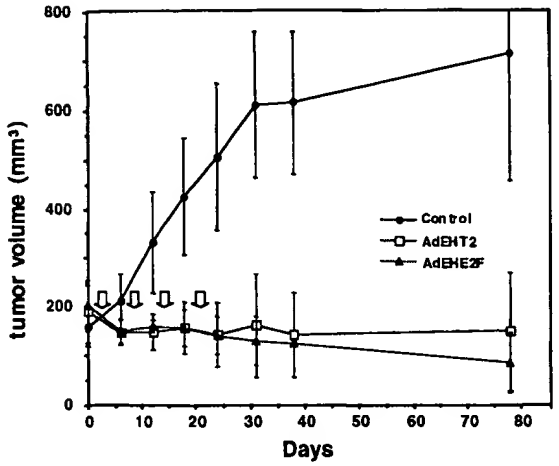

FIG. 10. Antitumor effect of AdEHT2 and AdEHE2F *in vivo*. Human tumor xenografts were established in estrogen-treated female nude mice by injection of BT-474 cells into the mammary fat pads. Mice were injected intratumorally with either vehicle alone (solid circles), 5×10^8 PFU of AdEHT2 (open squares), or 5×10^8 PFU of AdEHE2F (solid triangles) ($n = 5$). The treatment was administered for three consecutive days per week during the first 4 weeks (open arrows), and then the mice were left untreated. The significance of the differences observed between control and treated groups was analyzed by Student p test.

whose expression can be activated under different circumstances. The core hTERT promoter used in AdEHT2 contains AP2, E-box, Myc, and Sp1 binding sites (Horikawa *et al.*, 1999; Takakura *et al.*, 1999; Wick *et al.*, 1999). The E2F-1 promoter used in AdEHE2F contains MBF-1, Sp1, NF-κB, and E2F binding sites (Hsiao *et al.*, 1994; Neuman *et al.*, 1994). Second, both promoters can be potentially stimulated by E1A, either directly or as a consequence of the activation of the cell cycle. Finally, although the E4 region is believed to be necessary for efficient virus replication because the E4-deleted adenoviruses are attenuated, it is possible that low levels of expression of the E4 open reading frames (ORFs) can support replication if the rest of the transcription units of the virus are activated. A combination of these facts might explain the observation that the AdEHT2 virus was not attenuated in the telomerase-negative IMR-90 cells when they were infected under hypoxic conditions. Similar results were obtained with Saos-2 cells, a human osteosarcoma cell line that shows low telomerase activity (Zhang *et al.*, 2000; and data not shown). Although previous characterization of the hTERT core promoter suggested that it was able to provide negative regulation in telomerase-negative cells (Takakura *et al.*, 1999), it is possible that some key silencer is missing in this short fragment. Therefore, we cannot rule out the possibility that a longer regulatory region could restrict replication of the virus.

Although most of the previous considerations also apply to the E2F-1 promoter, the fact is that we saw attenuation of the AdEHE2F virus in cells with a low level of E2F activity, such as nontransformed cells (IMR-90) maintained in serum-free medium. This could be a useful feature of this CRAD, because these conditions apply to most somatic cells. The majority of cells in the connective tissue, for instance, are not hypoxic and show low levels of E2F transcription factors because they do not proliferate continuously. Therefore, it might work as a barrier for the proliferation of AdEHE2F. Many cells in a solid tumor such as breast cancer are also quiescent, but if they are infected by AdEHE2F, as long as they are hypoxic or express ER, the E1A region of the virus will be expressed, and this may activate the E4 region. Using the modified adenovirus backbone that we have constructed, different combinations of promoters can be tested in order to optimize the CRADs. The EREs can be avoided to prevent possible side effects in normal ER^+ tissues; a mutated E1A that does not bind to pRB can be tested to avoid activation of the E4 region (Johnson *et al.*, 2002); artificial minimal promoters can be placed in the E4 region to achieve a genuine second level of regulation, and newly characterized tumor-specific promoters for particular malignancies can be readily incorporated in the system. The goal in the design of CRADs is to achieve an optimum balance between attenuation in normal tissues and full cytolytic activity in tumors. All the measures described above can contribute to achieve this goal. In the case of AdEHT2 and AdEHE2F, the relatively low expression of E4 proteins might be the reason why these viruses are less efficient than wild type. Still, the E2F-1 promoter used in AdEHE2F contributed to the attenuation of the virus in normal cells. Combining this promoter with HREs might enhance the expression of E4 under hypoxic conditions, and increase the cytotoxicity of the virus without affecting its regulation. Finally, the new CRADs that we describe here have shown antitumor activity *in vivo*. They were able to block the growth of human tumor xenografts in nude mice, but they were not able to completely eradicate them in most of the animals. There may be physical barriers that limit the dissemination of the virus in the tumor mass. This problem should be addressed in order to enhance the oncolytic potential of these agents. AdEHE2F was well tolerated when administered intratumorally at doses that achieved a significant antitumor effect. It is less toxic than the wild-type adenovirus after intravenous administration, in terms of both mortality and liver toxicity. In fact, most of the mice that died from high doses of AdEHE2F had only moderate elevations of the ALT enzyme. At present, it is not clear if this reflects the existence of a different dose-limiting target organ. Despite the tight regulation of E1A expression observed in cell culture assays, this virus was still more toxic than a conventional E1-deleted, replication-deficient adenoviral vector. Strategies to achieve targeted infection, combined with the regulation of viral replication, will be necessary to obtain an optimum balance between safety and efficacy of CRADs.

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NOTE ADDED IN PROOF

During the preparation and evaluation of this manuscript, two new CRADs using the E2F-1 promoter have been described by

independent groups (Johnson *et al.*, 2002; Tsukuda *et al.*, 2002). Their results strongly support the use of this promoter to achieve cancer-specific replication of adenoviruses.

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Address reprint requests to:
Dr. Michael F. Clarke
Department of Internal Medicine-Hematology/Oncology
University of Michigan Comprehensive Cancer Center
1500 E. Medical Center Drive
4310 CCGC/0936
Ann Arbor, MI 48109

E-mail: mclarke@umich.edu

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